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Comparative Biology of Intracellular Parasitism

JAMES W. MOULDER

Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

There are more ways to choke a cat than feeding it hot butter

INTRODUCTION

The dictionary (150) says that the comparative approach is ". . . characterized by the comparison of things that have developed divergently from a common origin or of things that have developed convergently from different origins or of both . . . ." Therefore, a comparative examination of intracellular parasitism means asking how the present state of affairs came to be. It means looking at this highly successful way of life from an evolutionary point of view, which, according to Julian Huxley (209), is to ask of each structure or process examined not only "What is it?" and "What does it do?" but also, "Where did it come from?"

Of the several kinds of long-term associations between unlike organisms (390), the parasitic relationship is most simply defined as a food-getting habit in which one organism, the parasite, uses a second organism, the host, as a source of food. Intracellular parasites spend most of their lives within host cells. Obligate intracellular parasites have adapted so well to life inside cells that they no longer multiply outside of them, whereas facultative intracellular parasites still multiply in nonliving media. However, since most facultative intracellular parasites occupy intracellular habitats within their natural hosts, the distinction may not be terribly important.

I have prepared this review on the framework of several assumptions. The inside of a potential host cell is a hostile, uninviting environment (282, 283). Nevertheless, at many times in the evolutionary past, organisms of diverse origin have adapted to intracellular habitats (284). This is because host cells represent largely unexploited food sources, so that, if a parasite adapts successfully to an intracellular niche, it may thrive unimpeded by interspecies competition. Because the concept of unity in biochemistry implies that, at first approximation, the insides of all cells are more or less alike, evolutionary transition to intracellular parasitism may be looked on as the solution of a set of common problems (284): (i) how to get inside of host cells; (ii) how, once inside, to keep from being killed; (iii) how to multiply intracellularly; (iv) how to maintain host functions essential for parasite multiplication; (v) how to get new generations of parasites out of the host cells in which they were made; and (vi) how to get from old host cells to new ones. Since these problems were confronted at different times by organisms of unlike phylogeny, few general solutions have emerged, and the comparative analysis of intracellular parasitism consists mainly of identifying lines of both convergent and divergent evolution in which similar ends have been achieved by different means.

This review does not pretend to be an encyclopedia of intracellular parasitism. The host parasite systems I have described here are ones with which I am familiar and about

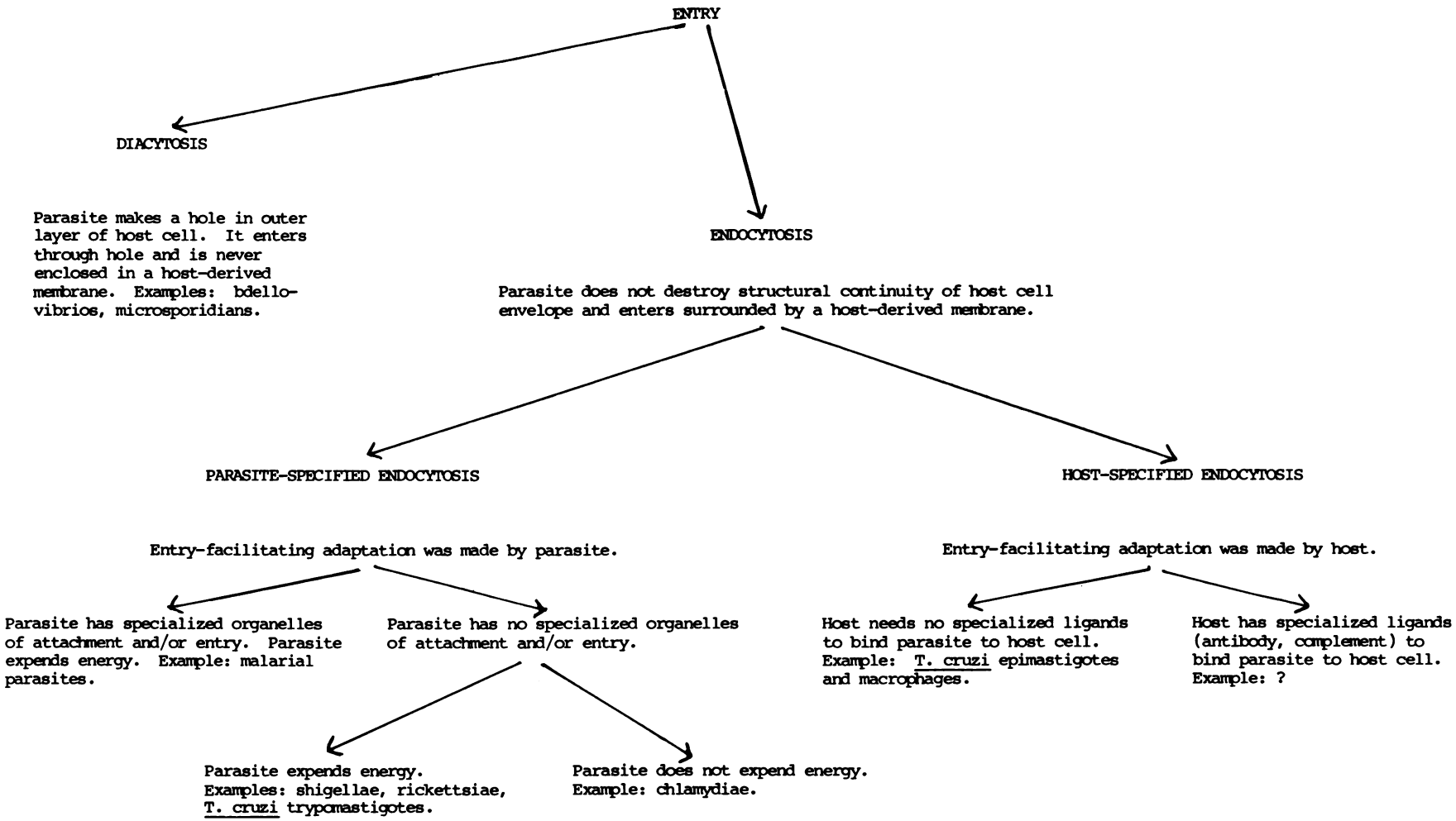

FIG. 1. How intracellular parasites get inside host cells.

which information of comparative value is available. The interaction of intracellular parasites with immune hosts has not been considered (for discussion of the voluminous literature on this subject, see reference 106). Most of the time, I will be concerned with the interaction of procaryotic and eucaryotic intracellular parasites with the cells of vertebrate hosts. However, when comparison appears to be enlightening, interactions in which the parasite is a virus and the host is an invertebrate animal, a plant, or even a procaryote will also be discussed. Because many intracellular parasites cause serious disease in humans and their domestic animals, the medically oriented investigator is in danger of becoming so preoccupied with these parasites alone that he forgets that intracellular parsitism is ubiquitous and that illuminating and instructive phenomena may be found in unexpected places.

ENTRY

Entry into host cells is the activity of intracellular parasites that lends itself most readily to the comparative approach. The translocation of an intracellular parasite from the outside to the inside of a host cell is a dramatic event with a clear beginning and end that may be described and quantified with relative ease, but as the parasite goes deeper into its host cell and further into its reproductive cycle, there is generally less information and more confusion.

Classification and Terminology of Entry Mechanisms

A few intracellular parasites make holes in the envelopes of their host cells and a few rely entirely on endocytosis by host cells to achieve their entry, but most of them enter by mechanisms requiring some kind of participation by both host and parasite. Description of entry mechanisms requires a classification and terminology. "Entry" will be used inclusively and noncommitally to describe all movements of parasites from the outside of the host cell to the inside. These movements will be classified in a hierarchy of six dichotomies. Figure 1 diagrams this classification and gives examples of each category, each of which will later be discussed in some detail.

The most direct way for one organism to get inside another is to punch a hole and go in through the hole. Since a term to describe this mode of entry is not available, I suggest "diacytosis." The Greek prefix *dia* means *through*, particularly with the connotation of *through and out on the other side*. Diacytosis has the additional advantage of parallel construction with its opposite "endocytosis" (a relatively new word itself) which was constructed from the Greek prefix *endo* meaning *in*, with the strong connotation of *taking in*, and the self-explanatory suffix, *-cytosis*. From the point of view of this discussion, the distinguishing feature of endocytosis is that the parasite enters in a host-derived membrane without disrupting the integrity of the host cell. How the parasite manages to pull off this seemingly contradictory trick is the basis of all subsequent bifurcations in the classification.

Host cells do not appear to internalize intracellular parasites readily unless either host or parasite has made some evolutionary adaptation that facilitates uptake. The basis of the next dichotomy is which partner in the host-parasite union has made the adaptation. Thus, I will distinguish between parasite-specified and host-specified endocytosis (73). This brings up another fuzzy but still useful dichotomy, that of professional and nonprofessional phagocytes (332). Cells other than the neutrophils and the monocytes of the blood and the macrophages of the tissues are regarded as nonprofessional phagocytes. These cells are not usually very good at endocytosis, and when a nonprofessional phagocyte efficiently takes up an intracellular parasite, it is almost always because the parasite has made an adaptation that promotes entry; that is, it is an example of parasite-specified endocytosis. As will be seen, such adaptations may be active, as in rickettsiae, or passive, as in chlamydiae. On the other hand, the professional phagocyte embodies a number of adaptations that make it capable of ingesting with ease a wide variety of encountered particulates. When an intracellular parasite is the object ingested, we have an example of host-specified endocytosis.

Parasite-specified endocytosis may be split into two types according to the presence or absence of specialized organelles of attachment and entry, and it is useful to subdivide the latter type further on the basis of whether or not the parasite expends energy in getting inside its host cell. Host-specified endocytosis, uptake of intracellular parasites by professional phagocytes, may be separated in a somewhat parallel way into a type that depends on specialized ligands (antibody or complement) to bind parasites to the phagocyte and one that does not. There is good reason to believe that the molecular mechanisms of parasite-specified and host-specified endocytosis are not the same. In particular, the host-derived membrane that surrounds the ingested parasite may be specifically modified in parasite-specified uptake.

The overriding consideration in drawing up this classification was the relative contribution of host and parasite to entry. A line drawn through Fig. 1 from upper left to lower right roughly approximates a gradient down which host activity increases and parasite activity decreases.

Modes of Entry

Bdellovibrios. The soil-inhabiting bacteria of the genus *Bdellovibrio* (65, 346, 350, 393, 394) are paradigms of diacytosis. The extremely small and highly motile bdellovibrio "attacks" a susceptible bacterial cell, makes a hole in the cell wall, and goes in through the hole to lodge in the periplasmic space. There, it immediately kills its host, breaks down the host macromolecules, and uses them to produce a brood of parasites which are released when the empty shell of the parasitized bacterium lyses. It has been argued that, since bdellovibrios lie intramurally and not intracytoplasmically, they are not intracellular parasites at all. One can even argue further that the bdellovibrios are not even parasites but are instead predators, much like the arthropod parasitoids (174). However, the bdellovibrios provide fascinating material for a comparative biology of intracellular parasitism precisely because they are so peculiar.

In a mixture of parasites and potential hosts, the bdellovibrio moves about at high velocity (100 cell lengths per s), propelled by a long, thick polar flagellum. It collides violently with both susceptible and nonsusceptible bacterial cells, but it attaches permanently only to susceptible ones. The point of collision and attachment is always the end opposite the flagellum. Some observers (364, 373) believe there are specialized organelles of attachment and penetration at that end, but others (1, 66) doubt their existence. Immediately after a productive collision, the bdellovibrio spins rapidly about the point of attachment in a arm-in-socket-like movement. The host wall bulges out, an enlarging "pore" appears, and within minutes the bdellovibrio squeezes in through the hole, perhaps propelled by its flagellum. The cell wall closes up behind the invader to leave a scar at the entry site, the cytoplasmic membrane separates

from the wall, and the bdellovibrio ends up in an intramural location.

Here, the host is clearly passive and the parasite active. Bdellovibrios enter ultraviolet (UV)-killed (425) and heat-killed (183) hosts at undiminished rates, but anything that inhibits their motility (395, 425) or their ability to synthesize protein (395) also inhibits attachment and entry. Just how the hole in the wall is made is not entirely certain. The bdellovibrio probably uses both mechanical (391, 393) and enzymatic (113, 207) forces. A number of bdellovibrio enzymes expressed at time of attachment probably facilitate formation of the entry pore and stabilization of the host bacterium's cell envelope (401, 402, 403) (see also section, "Multiplication").

Microsporidia. The microsporidia provide another equally good but more complicated example of diacytosis (62). Microsporidians are protozoa of the subphylum *Microspora*. They are distantly related to malarial parasites, toxoplasmas, and coccidians, all of which are sometimes loosely referred to as sporozoans. There are over 500 named microsporidian species, making them one of the largest groups of parasites. All are obligate intracellular parasites, their hosts range from protozoa to people, and they frequently kill their hosts. Pasteur discovered the first microsporidian almost 150 years ago, *Nosema bombycis*, the agent of silkworm disease.

Details of the microsporidian life cycle vary widely, depending on the particular host and parasite (427). Starting with an extracellular spore, its sporoplasm (nucleus plus a little cytoplasm) is injected into a host cell through an evaginating polar tube. Intracytoplasmically, the sporoplasm then develops into a meront (a large vegetative form), the meront divides repeatedly, and its progeny are distributed to other cells of the host by unknown means. One or 2 days after initial infection of the host, some of the meronts undergo sporogeny to produce a new generation of spores which are released extracellularly to complete the life cycle.

Most of the microsporidian spore is filled with a complex apparatus whose function is to inject the sporoplasm into a new host cell (426). On appropriate stimulation, water enters the spore, its internal hydrostatic pressure rises, and the instrument of injection, the polar tube, is turned inside-out with enough force to penetrate the cytoplasmic membrane of the target (249). Then the sporoplasm is transferred from the spore through the polar tube into new host cell. The penetrating power of the evaginating polar tube is so great that it may go right through other spores in its path. When the host cell's cytoplasmic membrane is punctured by the polar tube, the two structures fuse, thus preventing leakage of the host's cytosol. Although the penetration event itself is well documented by light (440) and electron (440, 442) microscopic observation, little is known about the stimulus that triggers it, other than that displacement of Ca^{2+} is involved (441). How does the microsporidian spore recognize a susceptible host cell, and how does it avoid premature or nonproductive discharge of its sporoplasm? These questions cannot be answered at the pesent time.

Malarial parasites. Malarial parasites furnish the best-understood example of parasite-directed endocytosis (32, 33, 134, 236, 367). The host cells are erythrocytes, perhaps the most metabolically limited and least phagocytically competent of all host cells inhabited by intracellular parasites, and the parasites have evolved complex organelles of entry. Malarial parasites (141) belong to the genus *Plasmodium* which is a part of the subphylum *Apicomplexa*, along with a number of other obligately intracellular protozoa such as *Eimeria* and *Toxoplasma*. These sporozoans enter host cells by mechanisms similar, but not identical, to those used by malarial parasites. Reptiles, birds, and mammals are hosts for many different species of *Plasmodium*, but most of the work on mode of entry has been done with the monkey parasite *Plasmodium knowlesi*. However, enough has been done on entry mechanisms in several other species of *Plasmodium*, human and otherwise, to suggest that they do not differ radically from that of *P. knowlesi*.

Malarial parasites pass through a complex life cycle that involves asexual and sexual stages, a mosquito and a vertebrate host, and two distinct phases in the vertebrate host, one occurring in the liver and the other in the bloodstream (141). However, of the several kinds of plasmodium-host cell encounter needed for each turn of the life cycle, only one has so far been studied: the entry of a merozoite liberated from one erythrocyte into another. This is not because this particular host cell-parasite interaction is so important: it is the only one it has been technically feasible to study.

The invasive blood form is the merozoite. Along with the usual organelles such as nucleus and ribosomes, the merozoite contains a number of subcellular structures that appear to play specific roles in attachment and entry. Merozoites adhere to their host cells by means of a thick cell coat (33, 272). This cell coat, which arises during schizogony and is immunologically of parasite origin, is an acidic glycoprotein that surrounds the merozoite with erect filaments (94 by 20 nm). Although any portion of the merozoite surface sticks to erythrocytes, adhesion is succeeded by invasion only when attachment is by means of the apical complex, a structure made up of polar rings, which give the complex its shape, and the interconnected secretary vesicles (rhoptries and micronemes) (32).

When the filamentous coat of the merozoite attaches to the plasma membrane of the erythrocyte at the site of the apical complex, there immediately ensues a spasmodic bending of the erythrocyte about the merozoite, the erythrocyte membrane invaginates, and the merozoite moves with a rotating motion into the resulting cavity (34, 103, 234, 272). The host cell membrane then fuses behind the merozoite so that it lies within the erythrocyte, surrounded by a membrane of presumably host origin (253). During these movements, which take less than 1 min, the merozoite coat is modified so that it can no longer be detected and the rhoptries and micronemes appear to be emptied of their contents (5). There is a fundamental difference between this entry mechanism and the two already described. The plasma membrane is never breached. With considerable help from the invading merozoite, the host cell engulfs the parasite and seals it off in a vacuole.

Despite much effort to find them out, the mechanisms responsible for the series of events I just described remain obscure. It is likely that the invagination of the erythrocyte membrane at point of contact with the apical prominence is brought about at least in part by the action of a protein secreted by the rhoptries (220, 221, 335). It has been postulated that this protein is similar or identical to the protein found in the dense granules of *P. lophurae* which is unusual in that 70% of its residues are histidines. When the isolated histidine-rich protein is added to erythrocytes, it cups them at low concentration and lyses them at high. Unfortunately, the protein present in the rhoptry has not been identified or isolated. The erythrocyte membrane is altered during plasmodial entry as evidenced by loss of membrane proteins and the appearance of membrane-bound channels leading into the cytoplasm, but it never breaks down. The source of

the energy that moves the nonmotile merozoite into the invaginated cavity in the erythrocyte has not been identified, and the origin of the signal for fusion of the erythrocyte membrane behind the entering merozoite is not known. Entry, but not attachment, of the malarial parasite is inhibited by treatment of merozoites with cytochalasin B (273).

Several lines of evidence suggest that merozoites and erythrocytes recognize each other by means of specific receptors on the surface of each reactant. In mammalian malarias, the kind of host cell invaded is severely limited; only reticulocytes, mature erythrocytes, and platelets are susceptible. Although initial contact of the merozoite with the erythrocyte is by means of its filamentous coat, there must also be additional receptors in the region of the apical prominence, but their chemical nature is unknown. Two major surface proteins of the human erythrocyte have been implicated as possible receptors for *P. falciparum*. Erythrocytes deficient in glycophorin resist parasite entry (315), antibodies to glycophorin (319) or the purified protein itself (423) block invasion, and surface proteins of *P. falciparum* bind to glycophorin (320). However, liposomes containing band 3 also prevent infection of human erythrocytes with *P. falciparum* (307). Erythrocyte receptors for the human malaria *P. vivax* may be related to the Duffy blood group antigens (275). The evidence for such a relation is that individuals of West African heritage usually do not have the Duffy antigens Fy^a, Fy^b, and Fy^3 and are insusceptible to infection with *P. vivax*, whereas Duffy-positive people are readily infected.

Trypanosoma cruzi. *T. cruzi*, the agent of Chagas disease, belongs to the subphylum *Sarcomastigophora*, superclass *Mastigophora* (the flagellated protozoa). Unlike other members of the genus *Trypanosoma*, such as the agents of African sleeping sickness, *T. cruzi* has a prominent intracellular stage in its life cycle (55). True bugs of the family *Reduviidae* (assassin bugs) are infected by feeding on individuals containing motile trypomastigotes in their blood. In the hind gut of the bugs, the trypomastigotes develop into epimastigotes which in turn given rise to a new generation of trypomastigotes which are shed in bug feces to enter new hosts by entering the bite wounds or by penetrating the mucosa of the mouth or eye, the favorite biting sites of the assassin bugs. Once in the bloodstream of the human hosts, they do not multiply but instead invade a variety of host cells such as heart muscle cells, endothelial cells, and macrophages. Then, within the intracellular habitat, they differentiate into nonmotile amastigotes which multiply extensively to produce more amastigotes and some new trypomastigotes as well. Both forms enter new host cells, and, in addition, the trypomastigotes infect more reduvid bugs to complete the life cycle.

Trypomastigotes enter mouse macrophages, HeLa cells, L cells, and calf fibroblasts, whereas epimastigotes enter macrophages but only attach to the other potential host cells (303). Amastigotes are infectious for macrophages and other cultured cells, but their interaction with these cells has not been studied in detail. Endocytosis appears to be the mechanism of entry. Host and parasite membranes do not fuse, and host cell membranes do not dissolve at point of parasite contact. Cytochalasin B, an inhibitor of microfilament function (94), inhibits the ingestion of epimastigotes (9, 303), trypomastigotes (303), and amastigotes (262).

The nature of the receptors on mouse macrophages that bind either epimastigotes or trypomastigotes is not known. Treatment of macrophages with proteases abolishes both attachment and ingestion of culture forms (303) but not bloodstream forms (8). Receptors for the C3b component of complement and the Fc portion of the antibody molecule do not appear to be involved (8, 303), but fibronectin, a ubiquitous mammalian cell surface protein, binds specifically to trypomastigotes, and antibody to fibronectin blocks the entry of trypomastigotes into rat fibroblasts (312). Protease treatment of mouse macrophages or L cells also inhibits the uptake of amastigotes from mouse spleen (262).

Shigella flexneri. The genus *Shigella* comprises four species of gram-negative, nonmotile bacteria (family *Enterobacteriaceae*) that produce acute diarrheal disease in humans (354). Although shigellae grow in simple synthetic media and are therefore to be considered facultative intracellular parasites, their ability to produce diarrheal disease depends on penetration of the epithelial cells of the colon wall, multiplication therein, and spread to other cells (127, 128, 232). The entry of *S. flexneri* into potential host cells has been studied more thoroughly than that of the other *Shigella* species.

There is no evidence for the participation of pili or other specialized organelles of attachment in the entry of *S. flexneri* into host cells (144). However, treatment of *S. flexneri* with UV light, heat, or the antibiotic kanamycin interferes with its entry into the Henle 407 line of human intestinal epithelial cells in vitro (161), which is good evidence for the active participation of shigellae in host cell entry. Although the Henle 407 cells are nonprofessional phagocytes, they internalize *S. flexneri* by a mechanism strongly resembling phagocytosis, and uptake of *S. flexneri* is inhibited by treatment of host cells with cytochalasin B or inhibitors of energy metabolism known to inhibit phagocytosis in macrophages (162). It has been concluded that virulent shigellae induce their uptake by host cells (161) which, in the terminology of Fig. 1, would constitute another case of parasite-directed endocytosis.

Genetic analysis has shown that virulence in *S. flexneri* requires the participation of all of a number of chromosomal loci, but the nature of their participation is largely unknown (144). At least one locus seems to be involved in the synthesis of lipopolysaccharides with a particular O-repeat unit (144). Virulent strains of *S. flexneri* also contain a 140-megadalton plasmid (359). Strains lacking this plasmid fail to enter HeLa cells and gain this ability when the plasmid is introduced. Anucleate minicells of *S. flexneri* containing the virulence plasmid enter HeLa cells and synthesize several outer membrane polypeptides, one or more of which may well participate in the binding of *S. flexneri* to host cells (163).

S. sonnei, *S. flexneri*, and enteroinvasive *Escherichia coli* also contain entry-associated plasmids in the 120- to 140-megadalton range. Although the three plasmids are clearly different, they share enough homologous sequences to suggest that they have evolved from a common ancestor (163).

Rickettsiae. Members of the genus *Rickettsia* are small, gram-negative, obligately intracellular bacteria (445, 446, 449). They cause a number of important human diseases in which the etiological agents are spread from one host to another by arthropod vectors. In their natural hosts, rickettsiae preferentially enter the endothelial cells lining the small blood vessels, and most of the host damage incurred in rickettsial infections stems from the parasitization of these cells. One of the first important studies of the entry of any nonviral intracellular parasite into host cells was carried out with the agent of scrub typhus, *Rickettsia tsutsugamushi*, and mouse lymphoblasts (86), but in recent years the host-parasite system consisting of *R. prowazekii*, the agent of louse-borne typhus, and mouse fibroblasts (L cells) has been

more frequently studied. The two rickettsial species appear to get inside of host cells by similar mechanisms, and the following description of rickettsial entry draws upon results obtained with both cell culture models.

R. tsutsugamushi and *R. prowazekii* readily enter professional and nonprofessional phagocytes both in vivo and in vitro by a mechanism resembling phagocytosis. They are internalized within a phagosomal membrane from which they quickly escape (22, 341), and their internalization is inhibited by cytochalasin B (436). Entry of rickettsiae into host cells is achieved by the interaction of viable rickettsiae with viable host cells. When host cells are made nonviable with heat or UV light, they do not ingest infectious rickettsiae (436), and, when the rickettsiae are similarly inactivated, they do not enter host cells (436). Oxytetracycline, which is rickettsiocidal, also inhibits the entry of *R. tsutsugamushi* (85).

Several activities of *R. tsutsugamushi* and *R. prowazekii* are dependent on a constant supply of glutamate which is oxidized with concomitant phosphorylation of adenosine diphosphate (ADP) (47). Host cell entry is one of these activities. When either the scrub or louse-borne typhus agent is deprived of glutamate, its ability to get inside host cells is drastically reduced (49, 86). All of these observations fit the conclusion that rickettsiae enter host cells by parasite-directed endocytosis.

Another glutamate-dependent in vitro activity of *R. prowazekii* is the lysis of the erythrocytes of a number of mammalian species, those of the sheep being most commonly used (49, 51). Lysis of erythrocytes by *R. prowazekii* furnishes an enlightening model system for studying entry of rickettsiae into host cells. Hemolysis occurs only when the rickettsiae attach to the erythrocyte membrane (333), and the effect of inhibitors on hemolysis closely parallels their effect on entry into L cells. *R. prowazekii* also attaches to erythrocyte stromata (464), thus furnishing an even simpler model in which it may be demonstrated that cholesterol is a part of the erythrocyte receptor for rickettsiae (334) and that hemolysis is accompanied by the release of free fatty acids and lysophosphatides by a phospholipase A whose origin (host or parasite) has not been determined (461, 463). It appears that a similar phospholipase A activity is also involved in the entry of *R. prowazekii* into L cells, although relatively high multiplicities of infection are needed to demonstrate the release of fatty acids and lysophosphatides from the L-cell plasma membrane (462). At this high ratio of rickettsiae to host cells, the integrity of host cell membranes is destroyed, and the L cells die before the start of rickettsial multiplication. Cytochalasin B-treated L cells are killed even more readily than untreated ones, thus demonstrating that only attachment, and not entry, is required for immediate rickettsial toxicity. Phospholipase A is also involved in entry of *R. rickettsii* into host cells (432).

Chlamydiae. Chlamydiae are gram-negative procaryotes that live as obligate intracellular parasites in a wide range of hosts (290, 361). There are two species, *Chlamydia psittaci* and *Chlamydia trachomatis*, which is further subdivided into the mouse, lymphogranuloma venereum (LGV), and trachoma biovars. *C. psittaci* is most frequently a parasite of birds and nonhuman mammals, although humans, cold-blooded vertebrates, and invertebrates may be infected. In contrast, *C. trachomatis*, with the exception of the mouse biovar, is strictly a human parasite. In mammals and birds, both species produce infections of the eye, the urogenital tract, the respiratory tract, the digestive tract, and probably other sites as well. There are two chlamydial cell types, the elementary body and the reticulate body, and the alternation of these two cell types constitutes the chlamydial developmental cycle. Elementary bodies never divide. Their role is to carry the infection from one host cell to another, where they reorganize into reticulate bodies which multiply by binary fission. Reticulate bodies do not infect new host cells. Instead, they reorganize into a new generation of elementary bodies to complete the developmental cycle. The outer membrane proteins of elementary bodies are more highly cross-linked with disulfide bonds than are those of reticulate bodies (see section, "Multiplication" for fuller discussion), and these structural differences may well account for many of the biological differences between the two chlamydial cell types.

Wherever they have been observed, morphological aspects of the entry of *C. psittaci* and *C. trachomatis* into both professional and nonprofessional phagocytes are similar (136, 187, 239). Single elementary bodies attach to the plasma membrane of the host cell, are enveloped by microvilli, and enter the host cell cytoplasm within the confines of a membrane-bound vacuole. In these respects, the chlamydial mechanism of entry does not differ significantly from those just described for *T. cruzi*, *S. flexneri*, and *R. prowazekii*, but in other respects it is different. First, chlamydial internalization by either professional or nonprofessional phagocytes is not inhibited by cytochalasin B (151, 229), although it is inhibited by cytochalasin D (438). Second, entry of chlamydiae into host cells is not prevented by inactivating the elementary bodies with UV light (70, 240) or by inhibiting their synthesis of macromolecules with antibiotics (230, 289).

Large inocula of chlamydiae are immediately toxic to host cells in vitro. When L cells (289) or macrophages (230, 239, 470, 471) are incubated with high multiplicities of elementary bodies, they lose membrane integrity and die long before onset of chlamydial multiplication. The resemblance of immediate chlamydial toxicity to that of rickettsiae is only superficial. Rickettsiae need only to attach to host cells and elaborate phospholipase A to be immediately toxic, but chlamydiae must both attach and enter. The actual penetration of the host cell cytoplasmic membrane by large numbers of chlamydial elementary bodies is the lethal event.

Although the several chlamydial species and biovars share a grossly similar cytochalasin B-insensitive entry mechanism, in their adaptation to different intracellular habitats, they have evolved different ways of getting inside host cells, so that a single uniform description of chlamydial entry is not possible. *C. psittaci* strains are generally more invasive than those of *C. trachomatis*. They have a wider host range and infect a greater variety of host cell types. Of the two *C. trachomatis* biovars that infect humans, LGV is more invasive than trachoma (42). These differences are reflected in cell cultures, where entry behavior has been almost exclusively studied. The 6BC and Cal 10 strains of *C. psittaci* have been used in most entry studies. Other *C. psittaci* strains may behave differently (96, 388). *C. psittaci* rapidly enters a variety of cultured cell types, including both macrophages and nonprofessional phagocytes. The LGV biovar behaves much like *C. psittaci* except that it infects macrophages very inefficiently (229, 230), and the trachoma biovar enters a limited range of cultured cells and only with some difficulty, a difficulty that does not prevent it from being a hugely successful human pathogen (362).

(i) *C. psittaci*. *C. psittaci* organisms attach to L cells at efficiencies 10 to 100 times greater than those observed with inert particles of similar size or noninvasive bacteria such as

E. coli K-12 (73). The rate of attachment is dependent on multiplicity of infection and temperature of incubation (71, 136). The efficiency of attachment decreases at high multiplicities, but saturation of the L-cell surface with respect to association with chlamydiae has not been achieved (71). The rapid rate and high efficiency of attachment of *C. psittaci* to nonprofessional phagocytes such as L cells have led to the hypothesis that this organism has evolved a surface ligand with high affinity for some normal, ubiquitous component of host cell surfaces (parasite-directed endocytosis) and that internalization of *C. psittaci* is the end result of specific binding between host and parasite receptors (73). The surface of *C. psittaci* is very hydrophobic and bears a net negative charge, due mainly to the presence of carboxyl groups (365, 422). The ability of *C. psittaci* to attach to L cells is destroyed by mild heating (70) and periodate oxidation (422) but it is unaffected by detergents, proteases, or antibiotics that inhibit synthesis of chlamydial macromolecules (73, 289). The capacity of L cells for binding *C. psittaci* is destroyed by proteases such as trypsin, and the regeneration of binding capacity is blocked by cycloheximide (70). L cells treated with periodate or acetic anhydride have reduced capacity to attach *C. psittaci*, but treatment of L cells with formaldehyde is without effect (181). Pretreatment of L cells with wheat germ agglutinin, a lectin specific for *N*-acetylglucosamine residues, has been reported to block attachment of *C. psittaci* (244), but this finding has not been confirmed by others (181). All in all, these results suggest that proteins, possibly glycoproteins, are essential parts of both hypothesized receptors, but efforts to link attachment to specific proteins on host and parasite surfaces have been inconclusive.

(ii) LGV biovar of *C. trachomatis*. In its interaction with cells in culture, LGV resembles *C. psittaci* in rate and in efficiency of association with host cells (73). As in *C. psittaci*–L-cell interactions, heating LGV reduces its capacity to attach to L cells, and trypsinization of L cells destroys their ability to attach LGV (73). These points of similarity suggest a single evolutionary origin for entry mechanisms in LGV and *C. psittaci*. However, since these two kinds of chlamydiae probably branched off the family tree early in chlamydial evolution (285), it is equally likely that their entry mechanisms originated independently and that resemblances represent convergent evolution within the intracellular habitat.

(iii) Trachoma biovar of *C. trachomatis*. In nature, the trachoma biovar causes disease only in humans, and the infection is largely limited to the columnar epithelial cells of the eye, the urogenital tract, and the respiratory tract. In culture, only a few cell lines make satisfactory hosts. Most investigations on entry mechanisms have been done with HeLa 221 cells and McCoy cells (a heteroploid mouse line). The trachoma biovar infects even these cells so lackadaisically that special entry-promoting procedures are used to achieve efficient isolation of these chlamydiae from clinical specimens. The most efficient of these procedures, centrifugation of the inoculum onto the host cell monolayer (102), appears to act by circumventing natural entry mechanisms rather than by enhancing their effectiveness (6, 15, 18, 240). For this reason, only studies in which the trachoma biovar was allowed to interact with host cells in the absence of centrifugation will be considered here.

The trachoma biovar associates with cells in culture in ways that set it apart both quantitatively and qualitatively from LGV and *C. psittaci*. Attachment and ingestion are much less efficient. At best, 10-fold-fewer host cells are infected in the absence of centrifugation than in its presence (240), and so few cells are infected by the new generation of elementary bodies released from initially infected cells that essentially a one-step infection is obtained. The same heat treatment that destroys the ability of *C. psittaci* and LGV to attach to host cells has no effect on the trachoma biovar, and digestion of host cells with trypsin does not keep them from associating with chlamydiae of the trachoma biovar (240). However, pretreatment of host cells with neuraminidase, which does not destroy their ability to attach LGV and *C. psittaci*, inhibits attachment of the trachoma biovar (45, 46, 231). Pretreatment of host cells with polycations such as diethylaminoethyl-dextran stimulates the entry of the trachoma biovar but not that of the other two kinds of chlamydiae (231, 384). These results suggest that, if specific chlamydial sites do indeed bind to specific sites on the host cell surface, then the receptors for both host and parasite are qualitatively different for the trachoma biovar than for the other chlamydiae. The observations that neuraminidase treatment of host cells blocks trachoma binding and that, under certain circumstances, treatment of host cells with wheat germ agglutinin has a similar effect (46, 386) suggest that sialic acid residues may be a part of the host cell receptor.

Because chlamydiae are so good at getting inside host cells with what appears on the surface to be a minimum expenditure of effort on their part, there has been considerable speculation as to how they do it. The zipper mechanism (152), originally proposed to explain immune phagocytosis by macrophages, and receptor-mediated endocytosis (314, 317), the process whereby a variety of proteins and peptides are taken into cells, have both been suggested as possible mechanisms of chlamydial entry (73, 385, 386, 438).

General Aspects of the Entry of Intracellular Parasites into Host Cells

Attachment of intracellular parasites to host cells. Relations between parasites and their hosts are seldom promiscuous. Even the most pugnacious of parasites, *Bdellovibrio*, attacks only gram-negative bacteria (395), and the most voracious of hosts, the macrophage, ingests some bacteria and leaves other alone. This selectivity on the part of both host and parasite is the basis of the phenomenon of recognition. It is generally assumed that intracellular parasites and host cells recognize each other through the interaction of complementary structures on their surfaces, although the specific examples of entry just presented reveal how little is known about these postulated receptors. They probably evolved by molecular tinkering (211), using the tools at hand for new jobs. The parasite receptor could have started out with some other function in the ancestral extracellular parasite, and, at some later time, a survival advantage to getting inside a host cell selected for parasite structures that bound more and more efficiently to host cells. In a similar fashion, the receptors on host cells must have evolved to perform functions that had nothing to do with host-parasite interactions and were then subverted to a new parasite-binding function. Except for the professional phagocytes, host cells have been under no pressure to evolve better ways of recognizing parasites. Indeed, the expected adaptation would be one of lessened reactivity, as may have occurred with erythrocytes and malarial parasites (275).

Some intracellular parasites are motile and some are not. The nonmotile ones must of necessity attach to host cells on random contact, but for the motile parasites there exists the

TABLE 1. Mode of entry and subsequent fate of selected intracellular parasites

Characteristic	Intracellular parasite											
	Bdellvibrios	Microsporidias	Plasmodias	*Toxoplasma gondii*	*Trypanosoma cruzi* (epimastigotes)	*Trypanosoma cruzi* (trypomastigotes)	*Leishmania donovani*[a]	*Mycobacterium tuberculosis*	*Shigella flexneri*	Rickettsiae	*Coxiella burnetii*	Chlamydiae
Parasite has specialized entry organelles	+	+	+	±[b]	0	0	0	0	0	0	0	0
Parasite makes hole in host cell envelope	+	+	0	0	0	0	0	0	0	0	0	0
Parasite enters nonprofessional phagocytes	−[c]	−	+	+	0	+	+	0	+	+	+	+
Parasite enters in host-derived vacuole	0	0	+	+	+	+	+	+	+	+	+	+
Parasite entry inhibited by cytochalasin B	−	−	+	+	+	+	+	−	+	+	+	0[d]
Parasite expends energy during entry	+	+	+	±	−	−	±	−	+	+	0	0
Parasite escapes vacuole after entry	−	−	0	0	0	+	0	0	+	+	0	0
Vacuole fuses with lysosomes	−	−	−	0	0	0	+	0	0	0	+	0

[a] Both promastigotes and amastigotes.
[b] Conflicting reports.
[c] No record of test.
[d] Entry inhibited by cytochalasin D.

possibility that they are led to suitable host cells by chemotaxis. Such an event has never been documented, but the demonstration that chemotactic stimuli guide cholera vibrios to the intestinal mucosa (132, 133) suggests that it might be well to look for a similar phenomenon with motile intracellular parasites, such as the malarial merozoites and sporozoites.

Diversity of entry mechanisms. Why attachment is followed by entry in some host-parasite systems and not in others (such as the *Mycoplasma*-host cell interaction [336]) remains a mystery. Entry is clearly a problem distinct from attachment, and in solving it intracellular parasites have shown so much evolutionary diversity that few generalizations can be made. Table 1 summarizes the entry characteristics of the seven intracellular parasites just described in the previous section, together with those of four others not previously mentioned (*Toxoplasma gondii*, *Leishmania donovani*, *Mycobacterium tuberculosis*, and *Coxiella burnetii*). The last two characteristics listed will be discussed in the next section.

It has been concluded that one of the most fundamental differences between eucaryotes and procaryotes is that the former engage in endocytosis and the latter do not (389). Such a difference goes a long way toward explaining why procaryotes have never been observed to serve as hosts for cytoplasm-dwelling organismal intracellular parasites, but no such eucaryotic-versus-procaryotic dichotomy emerges from examination of Fig. 1 and Table 1. Eucaryotic entry mechanisms cannot be uniquely set off from procaryotic mechanisms and vice versa.

Even among taxonomic groups of lesser breadth, common properties of entry mechanisms are hard to find (cf. the sporozoans *Microsporidium*, *Plasmodium*, and *Toxoplasma*; the trypanosomatids *Trypanosoma* and *Leishmania*; and the gram-negative bacteria *Shigella*, *Rickettsia*, *Coxiella*, and *Chlamydia* spp.). At present, evolutionary modification of an ancestral entry plan is discernible only within a single genus, for example, *Plasmodium* or *Chlamydia*. The family *Enterobacteriaceae* should be fertile ground for unearthing evidence of evolutionary change on a larger scale. This group of closely related gram-negative rods contains at least four genera of facultative intracellular parasites (*Escherichia*, *Shigella*, *Salmonella*, and *Yersinia*). It is unfortunate that the information needed to compare their modes of entry has never been assembled.

On the basis of the information at hand, it must be concluded that adaptations enabling one cell to get inside another have arisen many times among many different kinds of microorganisms and that there is no recognized example of evolutionary progression from primitive entry mechanisms to more advanced ones. Of the various entry modes defined in Fig. 1, parasite-directed endocytosis is the most frequent and the most varied, at least among the examples of Table 1. Such a result might have been predicted because the parasite is the point at which selection for efficient entry operates. By similar reasoning, it might also have been expected that diacytosis as a mode of entry would have been more frequently represented than it is. Perhaps the rather elaborate structural or enzymatic requirements of successful diacytosis have limited its occurrence, whereas endocytic mechanisms are already present, ready to be exploited, in potential host cells. Intracellular parasites must "direct" their host cells to engulf them in a number of different ways. For example, malarial parasites produce specialized entry organelles and secretions, but the other eucaryotic parasites of Table 1, although of equal size and complexity, do not. Again, among the procaryotes, rickettsiae and shigellae expend energy in gaining entrance to their host cells, but chlamydiae and *C. burnetii* do not. Although the entry of most of the intracellular parasites of Table 4 is blocked by cytochalasin B, an inhibitor of microfilament function (94), entry of chlamydiae is not interfered with. This puzzling observation may have been resolved by the recent finding that cytochalasin D, an even more powerful inhibitor of microfilament function, inhibits the entry of *C. trachomatis* (biovar LGV) into HeLa cells (438). It is possible that cytochalasin B is not an effective inhibitor of the microfilament-dependent endocytosis of objects as small as elementary bodies (313), although other explanations are not excluded.

When macrophages ingest intracellular parasites in the same way they phagocytize other microoranisms, this is host-directed phagocytosis (Fig. 1). Of the organisms of Table 1, *L. donovani*, mycobacteria, and *T. cruzi*

epimastigotes probably enter host cells in this way, although it is impossible to discount unequivocally all parasite contribution to entry. Unfortunately, the general lack of precise understanding of how macrophages attach and ingest objects in the absence of antibody or complement (378) also extends to intracellular parasites. Macrophages ingest some intracellular parasites more rapidly in the presence of antibody than in its absence, but I know of no case in which antibody or complement is an absolute requirement for entry.

Entry mechanisms in establishment of intracellular symbioses. Because I was familiar only with intracellular organisms that lead parasitic lives, it took me quite by surprise to learn a few years ago that the establishment of intracellular symbiosis between the freshwater coelenterate *Hydra viridis* and algae of the genus *Chlorella* resembles in many ways the phenomena I have just been describing (328). Algal symbionts are more readily ingested by the digestive cells of the coelenterate than are other algae or latex spheres. This superior efficiency of entry is destroyed by heating the algal symbionts or by treating the hydra cells with trypsin or concanavalin A. In the terminology of Fig. 1, the entry of *Chlorella* into *H. viridis* could be either "parasite directed" or "host directed" because both members of the symbiosis are supposed to benefit from the association. Other intracellular symbioses (see references 79, 93, 329) probably furnish still other instructive examples of how one cell gets inside another.

SURVIVAL

For an intracellular parasite to evolve a way of getting inside a desirable host cell without providing for its survival once inside would be like jumping out of the frying pan into the fire. Therefore, successful intracellular parasites have come up with a number of different strategies to avoid getting burned up in the intracellular habitat.

How Host Cells Kill Parasites Intracellularly

Ingestion of a potential pathogen that has made no provision for intracellular survival sets off a series of events that usually result in the death of that pathogen (111, 199). These events include an increased consumption of oxygen that leads to the appearance of hydrogen peroxide, superoxide radical, and other oxygen-derived radicals, together with activation of myeloperoxidase. Lysosomes fuse with the pathogen-containing phagosome and release their acid hydrolases (glycosidases, proteases, and lipases). Lysozyme, lactoferrin, and cationic proteins may also be active. Polymorphonuclear leukocytes exhibit all of these potential microbicidal activities, macrophages exhibit most of them, and nonprofessional phagocytes exhibit probably only the lysosomal enzymes.

Polymorphonuclear leukocytes rarely serve as hosts for intracellular parasites (some *Ehrlichia* species are exceptions [343]), but many different kinds of intracellular parasites thrive within macrophages, where they must successfully resist any and all of the many microbicidal mechanisms that macrophages possess (see reference 331), but the only survival mechanism that has been described in a wide enough variety of parasite-host cell combinations to permit a comparative approach is evasion of the lethal consequences of the fusion of lysosomes with parasite-containing phagosomes.

Evasion of the Lethal Consequences of Phagosome-Lysosome Fusion

Since lysosomes are almost universal constituents of eucaryotic cells, it is no wonder that intracellular parasites have evolved more than one way of dealing with them.

Adaptation to a host cell without lysosomes. The lysosome-free (173), mature, non-nucleated erythrocyte is the host cell for many *Plasmodium* species and other hemosporidia (141), as well as for members of the families *Bartonellaceae* and *Anaplasmataceae* (order *Rickettsiales*) (345), which are obligate or facultative intracellular gram-negative bacteria that cause serious and sometimes widespread disease in people and in a variety of wild and domestic mammals (344). Parasitism of a lysosome-free host cell is a wonderfully simple way to avoid lysosome-phagosome fusion. However, the overwhelming majority of these erythrocyte-dwelling parasites live in other, lysosome-containing cells at some stage in their life cycles or are thought to have descended from ancestors that first lived in non-erythrocytic cells. Therefore, primary adaptation of an extracellular parasite to an erythrocytic host, although an entirely logical strategy, has probably played no important part in the evolution of mechanisms for intracellular survival.

Escape from the phagosome. A second way for an intracellular parasite to avoid the consequences of lysosome-phagosome fusion is for it to escape from the phagosome soon after it enters the host cell. Two quite unrelated intracellular parasites, the protozoan *T. cruzi* and the procaryotic rickettsiae, have made this adaptation. There is some evidence that it may be even more widespread.

T. cruzi. When trypomastigotes of *T. cruzi* are taken into unstimulated mouse macrophages, they remain in phagosomes for at least 1 h after entry but are free of phagosomal membranes and multiplying in the cytoplasm at 24 to 48 h (271, 303). Epimastigotes, on the other hand, do not escape from phagosomes and are killed and digested (303).

Rickettsiae. Although the first observation of rickettsial escape from phagosomes was with the agent of murine typhus (*R. typhi*) in human peripheral blood monocytes (22), most subsequent observations have been on the scrub typhus agent (*R. tsutsugamushi*) in either peritoneal mesothelial cells of mice (nonprofessional phagocytes) (117) or polymorphonuclear leukocytes (professional phagocytes) (340, 341). In all of these host-parasite interactions, the rickettsiae enter host cells in membrane-bound vacuoles but are free in the cytoplasm a short time later, perhaps in as little as 30 min. It has been suggested that phospholipase A, which has also been implicated in alteration of the host cell plasma membrane during rickettsial entry, may also be responsible for dissolution of the phagosome membrane during rickettsial escape (462).

Resistance to lysosomal enzymes. Another way to avoid being killed and digested by lysosomal enzymes is to become resistant to their action. Several intracellular parasites, including *Yersinia pestis* (396), *Salmonella typhimurium* (75), *Coxiella burnetii*, and the genus *Leishmania*, have made this adaptation. The latter two parasites are discussed in detail.

Leishmanias. Leishmanias belong to the same family as *T. cruzi* (*Trypanosomatidae*) (2, 78). Humans and other vertebrates are the hosts for numerous *Leishmania* species. Most studies on intracellular survival have been done with the human parasites *L. donovani* and *L. mexicana*. *L. donovani* causes a severe and often fatal visceral leishmaniasis (kala

azar) in the tropics and near-tropics of both hemispheres. In its broad aspects, the life cycle of *L. donovani* resembles that of *T. cruzi*. People are infected by the bite of sand flies (*Phlebotomus*). Extracellular, flagellated promastigotes produced in the invertebrate host are phagocytized by macrophages in which they differentiate into nonflagellated amastigotes which multiply intracellularly. They are released to infect other macrophages or to be taken up in the blood meal of another sand fly, in which they give rise to a new generation of promastigotes, thus completing the life cycle. In culture, leishmanias enter both professional (41, 78) and nonprofessional (245) phagocytes by a cytochalasin B-sensitive mechanism (9).

It was first shown for the in vitro systems of *L. mexicana* and mouse macrophages (11) and *L. donovani* and hamster macrophages (80, 81) that amastigotes enter their host cells in phagosomes which fuse with secondary lysosomes and that the leishmanias survive and multiply in the resulting phagolysosomes. These results have been confirmed with other *Leishmania*-macrophage systems (246). Fusion of primary lysosomes with phagosomes containing amastigotes is also without effect on survival or multiplication of the parasite (77). That leishmanias behave similarly in vivo was shown by infecting hamsters with *L. donovani* and then isolating and observing infected macrophages from the hamsters' bone marrow (104).

How do leishmanias survive and multiply in the presence of lysosomal enzymes? There are several possible answers, not necessarily mutually exclusive. First, *Leishmania* spp. may have evolved an enzyme-resistant cell surface (80). Surface glycoproteins appear to be critical to this kind of adaptation (82). Second, *Leishmania* spp. may secrete enzyme inhibitors or inactivators (11). In support of this hypothesis, the excretion of carbohydrate-rich molecules bearing large negative charges has been observed in several *Leishmania* species (110). These excretory factors inhibit some, but not all, of the acid hydrolases of the lysosome. It has also been suggested that ammonia produced by the highly active leishmanial proteases may accumulate in the leishmanial phagolysosome, thus raising the pH and inactivating the lysosomal enzymes, which have exceptionally low pH optima (89, 330).

That leishmanias may not be completely indifferent to lysosomal enzymes is suggested by the observation that poly-D-glutamic acid inhibits the fusion of secondary lysosomes with *L. mexicana*-containing phagosomes (see reference 168) and that *L. mexicana* grows faster in poly-D-glutamic acid-treated macrophages than in untreated ones (10).

Coxiella burnetii. *Coxiella burnetii*, a gram-negative obligately intracellular procaryote, is the sole representative of a genus formally classified in the order *Rickettsiales* but without known phylogenetic kinship with other members of the order (29, 451). *C. burnetii* is of worldwide occurrence in ticks and in mammals, particularly in cattle, sheep, and goats. It is transmitted by aerosol from domestic animals to people, in whom it causes the disease called Q fever (which is seldom fatal but in a few cases causes severe endocarditis [29]).

C. burnetii has been grown in vitro in both professional and nonprofessional phagocytes (29). It appears to enter nonprofessionals such as L cells by a cytochalasin B-sensitive (O. G. Baca, personal communication), parasite-directed endocytosis (67), although the role of the entering parasite seems to be more passive than that of *Rickettsia*. Once inside its host cell, *C. burnetii* remains and multiplies within membrane-bound vacuoles which have the properties of phagolysosomes (7, 67, 68). They contain the lysosomal enzymes acid phosphatase and 5′-nucleotidase, and they have a pH of about 5.0.

How *C. burnetii* resists the attack of lysosomal enzymes is not known, but there is no doubt that this organism has become well adapted to the acidic environment of the phagolysosome. Intact cells of *C. burnetii* utilize glucose and glutamate very slowly at pH 7 (309, 310), but they transport and metabolize both substrates very vigorously at pH 5 and below (157, 158, 159). Furthermore, when the pH of *C. burnetii*-containing phagolysosomes is raised by the use of basic lysosomotrophic agents such as chloroquine, methylamine, and ammonium chloride, parasite multiplication is inhibited (158).

Prevention of phagosome-lysosome fusion. A final way to avoid the lethal consequences of phagosome-lysosome fusion is to keep it from happening at all. As with the other avoidance strategies, this one has been adopted by a phylogenetically dissimilar group of intracellular microorganisms, among them being *M. tuberculosis* (24), *C. psittaci* (136), *Legionella pneumonophila* (202), *Toxoplasma gondii* (215), and chorella-like algae (188, 304).

M. tuberculosis. The genus *Mycobacterium* (36) includes the agents of two major human diseases, tuberculosis and leprosy, as well as a number of other pathogenic and saprophytic species. *Mycobacterium* offers an interesting example of adaptive radiation. As members of the genus entered new habitats and filled new niches, they evolved different mechanisms of intracellular survival. The rat leprosy agent *M. lepraemurium* lives in phagosomes that have fused with lysosomes (19, 169), the leprosy bacillus *M. leprae* spends part of its intracellular residence in phagosomes and part free in the cytoplasm (23, 116, 277), and *M. microti*, a parasite of voles, and the human tubercle bacillus *M. tuberculosis* live in phagosomes that seldom provoke lysosomal fusion (24, 169).

Of all of these mycobacteria, the relation between *M. tuberculosis* and lysosomes has been investigated by far the most exhaustively. Although human-virulent strains grow, albeit very slowly, in artificial media of simple chemical composition, in their human hosts they multiply almost exclusively in macrophages (472). In vitro, *M. tuberculosis* readily infects macrophages, but information on its mechanism of entry is surprisingly limited, considering the diligence with which other aspects of this host cell-intracellular parasite relationship have been studied.

When mouse peritoneal macrophages ingest human-virulent *M. tuberculosis* inactivated by gamma radiation, virtually all the bacilli-containing phagosomes fuse with ferritin-labeled secondary lysosomes (24). In contrast, when macrophages ingest viable *M. tuberculosis*, signs of fusion are infrequent. Exposure to specific rabbit antiserum does not destroy infectivity, but when the antiserum-treated bacilli are ingested by macrophages, most of the phagosomes fuse with ferritin-labeled lysosomes (24). However, the antiserum-coated bacilli continue to multiply in the fused phagosomes (25), thus showing that macrophage lysosomal enzymes do not inhibit the growth of *M. tuberculosis*. It appears that this intracellular parasite has evolved not one, but two ways of evading the consequences of lysosomal fusion.

A likely explanation for the reluctance of lysosomes to fuse with phagosomes containing viable *M. tuberculosis* is that mycobacterial products interact with lysosomal membranes and render them nonfusible. These products are

probably the mycobacterial sulfatides, polyanionic trehalose glycolipids that are associated with virulence in *M. tuberculosis* (148). The sulfatides are readily taken up by lysosomes, and exposure of mouse macrophages to small amounts of sulfatides prevents or delays the fusion of lysosomes with yeast cells, which normally provoke a massive fusion reaction (149). Continued study of the effect of various substances on the fusion of labeled secondary lysosomes with phagosomes containing yeast cells revealed that other polyanions (dextran sulfate, poly-D-glutamate, suramin, and chlorite-oxidized amylose) also inhibit fusion (143, 168, 170, 172), whereas chloroquine and other secondary and tertiary amines promote it (170). Chloroquine reverses the fusion inhibition produced by polyanions or by viable *M. tuberculosis* itself (170). When chlorite-oxidized amylose is coupled with a fluorescent label, it combines only with lysosomes and not with phagosomes, indicating that the polyanionic site of action is the lysosomal membrane (143). The observation that polyanionic inhibitors of lysosomal fusion also inhibit saltatory lysosomal movements has suggested that these inhibitors act largely, but not necessarily exclusively, by limiting lysosomal movement, thus reducing the frequency of lysosomal-phagosomal collision (172).

Ammonia, which freely enters the lysosomes, has an unexpected effect on phagosome-lysosome fusion. In accordance with the effect of chloroquine and other amines, ammonia would be expected to promote fusion, but in actuality it inhibits it (147). This action of ammonia probably does not result from raising the intralysosomal pH, because chloroquine also raises the pH.

There is one set of observations that does not fit into a picture of fusion/nonfusion being determined solely at the lysosomal membrane. Phagosome membranes around newly ingested yeast cells are tightly applied (171). However, treatment with polyanions causes the membranes to loosen, and this loosening is prevented or reversed by chloramphenicol. This suggests that something is also being determined at the phagosome membrane.

It has been suggested that *M. microti* avoids phagosome-lysosome fusion in mouse macrophages by raising the intracellular level of cyclic adenosine monophosphate (AMP) (251), but comparable experiments with *M. tuberculosis* have not been reported.

C. psittaci. When mouse fibroblasts (L cells) ingest infectious elementary bodies of *C. psittaci* (Cal 10), electron microscopic observation reveals no fusion of lysosomes with chlamydia-laden phagosomes, and in isopycnic centrifugation of fractionated infected L cells, chlamydial phagosomes and lysosomes appear as two distinct peaks (136). Comparable observations have been made with the polyarthritis strain of *C. psittaci* growing in bovine spleen cells (407) and with an LGV biovar of *C. trachomatis* in hamster cells (BHK-21) (239). When *C. psittaci* is heat inactivated before its ingestion by L cells, lysosomes promptly fuse with the chlamydial phagosomes (136). L-cell phagosomes containing antibody-coated *C. psittaci* also fuse with lysosomes (136). Since infectivity may be recovered by diluting the antibody-coated chlamydiae, the crucial effect of antibody is probably modification of the elementary body surface, not destruction of infectivity. When L cells ingest *C. psittaci* in the presence of multiplication-inhibiting concentrations of chloramphenicol, there is no phagosome-lysosome fusion for at least 30 h (136), indicating that the ingested *C. psittaci* need to neither multiply nor synthesize protein to prevent fusion. Therefore, the studies on inhibition of phagosome-lysosome fusion in L cells suggest that "the ability of the chlamydial cell to prevent the host lysosomal response was afforded by some intrinsic architecture of the parasite already present at time of entry" (136).

This suggestion is supported by investigations on chlamydial inhibition of lysosomal fusion in mouse peritoneal macrophages. As followed with ferritin-labeled secondary lysosomes and electron microscopy, intact elementary bodies of *C. psittaci* (Cal 10) do not trigger phagosome-lysosome fusion, but heat-treated and antibody-coated chlamydiae do (471). Isolated cell walls of elementary bodies, which are ingested as rapidly as intact chlamydiae (244), do not provoke fusion of lysosomes with the phagosomes in which they enter macrophages, but if the walls are heated before ingestion, they no longer inhibit fusion (108). Although chlamydial reticulate bodies are noninfectious, they are ingested by macrophages, whereupon reticulate body-bearing phagosomes are fused with lysosomes (58). Thus, it appears that the feature of elementary body architecture responsible for fusion inhibition is absent from the reticulate body, although lysosomes are not observed to fuse with chlamydia-laden vacuoles long after all elementary bodies have differentiated into reticulate bodies (407). When macrophages are allowed to ingest mixtures of *C. psittaci* elementary bodies and either yeast cells or *E. coli*, inhibition of fusion with lysosomes is restricted to the chlamydia-containing phagosomes; there is no generalized, host cell-wide interference with fusion (107).

With *M. tuberculosis*, bacterial products, probably sulfatides, are thought to modify lysosomal membranes and thus inhibit fusion. In contrast, with *C. psittaci*, at some as yet undetermined point in the entry process, some element of the elementary body wall appears to modify the membrane of the phagosome in which it is contained. When the phagosome membranes are isolated from macrophages that have ingested either infectious or heat-killed *C. psittaci*, their protein content is very similar, but there are small differences (474). Whether or not these differences determine the intracellular fate of chlamydiae remains to be seen.

Legionella pneumophila. *L. pneumophila* is the best characterized of a group of closely related gram-negative bacilli that have been isolated from freshwater lakes and streams and from domestic water supplies (56, 200, 238, 404). *L. pneumophila* infects people by the aerosol route to produce two clinically distinct diseases, an often fatal pneumonia (legionnaires' disease) and a mild, nonpneumonic illness (Pontiac fever). The organism does not grow in ordinary bacteriological media, but it does grow in special media designed to meet its exacting, but not complex, nutritional requirements (see section, "Difference Between Obligate and Facultative Intracellular Parasites"). In cell culture, it lives as an intracellular parasite. The mode of entry of the legionnaires' disease agent into host cells has not been studied in detail, but it enters and grows in both professional (human monocytes [203]) and nonprofessional (human embryonic lung fibroblasts [469]) phagocytes.

In monocytes from peripheral human blood, *L. pneumophila* multiplies in phagosomes with membranes of novel structure; they become studded with host ribosomes 4 to 8 h after infection (201, 203). Lysosomes prelabeled with thorium dioxide do not fuse with phagosomes containing live bacteria, and there is no sign of acid phosphatase activity in these phagosomes (202). However, if the legionnaires' disease bacteria are killed with Formalin before being ingested by monocytes, phagosome-lysosomes fusion is not inhibited, and host ribosomes do not associate with phagosome membranes (201, 203). Erythromycin, which inhibits protein

synthesis by intracellular *L. pneumophila* (204), fails to reverse the inhibition of lysosomal fusion and does not interfere with the formation of ribosome-studded phagosome membranes (201, 202). When the bacteria are coated with antibody, inhibition of fusion is partially overcome, although the entry and intracellular multiplication of antibody-coated organisms is not hindered (202).

As with chlamydiae, some structural feature of the unaltered bacterial surface, already present at the time of entry, appears to modify the membranes of phagosomes containing *L. pneumophila*. However, the correlation between inhibition of lysosomal fusion and formation of the novel ribosome-studded phagosomal membrane suggests that this mechanism of fusion is different from those evolved by either *C. psittaci* or *M. tuberculosis*.

Chlorella-like algae. The *H. viridis-Chlorella* symbiosis resembles the bacterial pathogen-vertebrate host cell relation, not only with respect to entry, but also with respect to survival. Viable symbiotic algae are ingested by the gastrodermal digestive cells of the coelenterate, each algal cell within a single vacuole, and phagosome-lysosome fusion does not occur (188, 304). However, nonviable algae are quickly digested. The hydra-alga symbiosis takes on added interest with the report that *H. viridis* is the host for microorganisms strongly resembling chlamydiae, although without the genus-specific chlamydial antigen (305). These chlamydia-like organisms grow within vacuoles inside ectodermal epitheliomuscular cells where they do not provoke lysosomal fusion. However, in gastrodermal cells, organism-containing vacuoles fuse with lysosomes, and the chlamydiae-like organisms are digested. The many resemblances between the relation of *H. viridis* with its symbionts and parasites and the relation of intracellular bacterial pathogens with their vertebrate host cells may provide a wealth of interesting material for future students of the comparative biology of intracellular parasitism.

Diversity of mechanisms for evading phagosome-lysosome fusion. Mechanisms for evading phagosome-lysosome fusion have evolved on the same pattern as already described for entry mechanisms: the appearance in different intracellular parasites of different solutions to a common problem. Even when two organisms initially appear to handle the problem in the same way, as in inhibition of lysosomal fusion in *M. tuberculosis* and *C. psittaci*, further study shows that they achieve the same end by different means.

MULTIPLICATION

After an intracellular parasite enters a host cell, it must not only survive in the intracellular environment, but also exploit the resources of that environment for its own multiplication without destroying, at least not immediately, host functions vital to parasite multiplication. Reproduction of the parasite and preservation of host functions are, of course, simultaneous and inseparable events. However, I will attempt to separate them for ease of discussion, although I will not always succeed. First, let us look at the multiplication of some representative intracellular parasites and then see if there are any generalizations to be made.

Modes of Multiplication

Bdellovibrios. Multiplication of *Bdellovibrio bacteriovorus* in the intraperiplasmic space of a gram-negative bacterial host furnishes a model of simplicity against which other modes of intracellular multiplication may be profitably compared. There are no host defenses to overcome and no host functions to preserve because bdellovibrios kill their hosts and stop host metabolism soon after entry. *Bdellovibrio* multiplies in a closed system in which the parasite uses what host resources are available at the outset and no more, whereas the other intracellular parasites to be compared with *Bdellovibrio* grow in open systems in which parasite multiplication is probably dependent on the continued ability of the host cell to obtain nutrients from the extracellular environment and to convert them into forms that the parasite can use (350).

Host cells such as *E. coli* are lethally damaged soon after bdellovibrio attachment (259, 349, 424). Flagella stop beating, cytoplasmic membranes are damaged, respiration ceases, and synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) stops. Within 30 min, the infected *E. coli* changes from a rod to a sphere called the bdelloplast, the structural unit of bdellovibrio reproduction in which growth and multiplication of the parasite takes place in the space between the swollen wall and the contracted protoplast of the host (346). The entry-associated breakdown of peptidoglycan by glycanase (401) is terminated by the action of another bdellovibrio enzyme that hydrolyzes *N*-acetyl groups from the peptidoglycan (402), thus making it an unfit substrate for glycanase. Bdellovibrio enzymes also bring about the addition of diaminopimelic acid (356) and long-chain fatty acids (403) to the bdelloplast wall. Other bdellovibrios that subsequently encounter the bdelloplast cannot make holes in the deacetylated peptidoglycan, and so the first bdellovibrio on the scene does not have to share the resources of the host cell with latecomers.

Next, protected from outside interference and with the entry pore sealed (by mechanisms unknown) to prevent loss of solubilized host material, the bdellovibrio efficiently converts host macromolecules into parasite macromolecules. Bdellovibrios grow normally on heat-killed host cells (183) or in complex media supplemented with host cell extracts (198, 338), so it is unlikely that they depend on their hosts for either degradative or synthetic enzymes or for generation of metabolic energy. Some host macromolecules, notably several outer membrane proteins (99), may be transferred intact from host to bdellovibrio, but most often host polymers are broken down to monomers at rates consistent with the synthetic needs of the parasite. For example, host DNA is split into 500-kilodalton pieces by an endonuclease, and then an exonuclease hydrolyzes off deoxyribonucleoside monophosphates which are incorporated directly into parasite DNA (259, 351). Similar mechanisms probably exist for breakdown and resynthesis of RNA and protein (184). Double-labeling experiments show that monomers produced by breakdown of host polymers are incorporated directly into bdellovibrio polymers with little or no modification (184, 228, 259, 348).

The major source of energy for bdellovibrio multiplication is the adenosine triphosphate (ATP) generated by aerobic oxidation of amino acids, glutamate in particular, liberated by hydrolysis of host protein (185). This energy is utilized with exceptional efficiency (347), an efficiency much greater than that achieved by host-independent bacteria growing in rich media and actually approaching the theoretical limit expected of an organism using energy solely for the polymerization of monomers. The basis of this high efficiency is not fully understood. The direct conversion of host-derived monomers into parasite polymers, such as the use of host-derived nucleoside monophophosphates for synthesis of DNA and RNA (348), contributes to the observed efficiency but does not fully explain it (346, 350).

TABLE 2. Multiplication of intracellular parasites

Salient features	*Bdellovibrio*	*Coxiella*	*Rickettsia*	*Chlamydia*	*Plasmodium*
Experimental hosts	Gram-negative bacteria, usually *E. coli*	Chicken embryos, eucaryotic cell lines (L cells, Vero cells)	Chicken embryos, eucaryotic cell lines (L cells)	Chicken embryos, eucaryotic cell lines (L cells, HeLa cells)	Erythrocytes from infected birds and mammals. Erythrocytes cultured in vitro
Location of multiplying unit	Intraperiplasmic space	Phagolysosome	Cytoplasm (free)	Cytoplasmic vacuole	Cytoplasmic vacuole
Mode of division	Fragmentation of filament	Binary fission	Binary fission	Binary fission	Segmentation of multinuclear form
Yield (no. of new infectious units) per host cell	4 to 20	Up to 1,000	100 to 1,000	10 to 1,000	6 to 20
Length of reproductive cycle	4 h	Several days	3 to 5 days	2 to 3 days	1 to 3 days
Damage to host cells	Damage is immediate and lethal	Morphologically evident damage is minimal. Damage at a low multiplicity not well studied	At high multiplicity of infection damage is immediate and lethal. Damage at low multiplicity not well studied	At high multiplicity, damage is immediate and lethal. At low multiplicity, host cells may continue to multiply	Host is a nonmultiplying cell. Damage evidenced by alteration of erythrocyte membrane and breakdown of hemoglobin
Energy source	ATP from aerobic oxidation of amino acids liberated from host proteins	ATP from aerobic oxidation of glutamate, also glucose	ATP from oxidation of glutamate. No catabolism of glucose. Transport and limited use of exogenous ATP	No net synthesis of ATP from exogenous substrates. Transport and use of exogenous ATP for macromolecule synthesis	ATP from substrate-level phosphorylation accompanying glycolysis of glucose to lactate. Aerobic oxidation in dispute. Limited use of exogenous ATP
Independent synthesis of macromolecules	Yes. Protein, RNA, and DNA syntheses in heat-killed host cells	Yes. Protein, RNA, and DNA syntheses in host-free parasites	Yes. Protein synthesis in host-free rickettsiae. Protein and nucleic acid syntheses in cycloheximide-treated host cells	Yes. Protein and RNA syntheses in host-free chlamydiae. Protein, RNA, and DNA syntheses in cycloheximide- and emetine-treated host cells	Yes. Host-free plasmodia make protein, RNA, and DNA as shown by isotope incorporation and by limited growth and development
Phosphorylation level at which host nucleosides are incorporated into nucleic acid	Monophosphates from breakdown of host nucleic acids	Probably mono- and diphosphates from host pools	Mainly monophosphates from host pools. Host pyrimidine nucleosides not used	Triphosphates from host pools used to make RNA (and DNA?). Host pyrimidine deoxynucleosides not used	Purine nucleosides of host pools incorporated into nucleic acid; pyrimidine nucleosides are not. Phosphorylation level unknown
Special features	Host is a procaryote	Developmental cycle with endospore-like and vegetative cell types	Only *R. tsutsugamushi* does not need a CO_2-enriched atmosphere for growth. Developmental cycle not demonstrated	Developmental cycle with infectious and reproductive cell types	Parasite is a eucaryote. Metabolic studies limited to one stage in life cycle (erythrocytic stage)

While all these biochemical events are going on, a new generation of bdellovibrios is being made. The invading bdellovibrio, which has left its flagellum behind upon entry, becomes a filament which grows in length until the food supply is exhausted or DNA synthesis is terminated (346). At that time, flagella reappear, and the filament breaks up into unit cell lengths, about four for each *E. coli* cell and several times that number for larger hosts (109, 219). Lysis and release of progeny is brought about by the appearance of a new parasite enzyme that hydrolyzes the deacetylated peptidoglycan of the bdelloplast (401). The entire life cycle of *Bdellovibrio*, from attachment to lysis, takes about 4 h.

Enough is known about four other intracellular parasites, three procaryotes and one eucaryote, to make worthwhile comparisons of their multiplication with that of the bdellovibrios. The available information is summarized in Table 2 and discussed in some detail for each parasite.

Coxiella burnetii. In cultures of L cells, green monkey kidney (Vero) cells, and several macrophage-like lines derived from mouse tumors, *C. burnetii* multiples by binary fission in enormous cytoplasm-filling vacuoles that have the properties of phagolysosomes (7, 28, 67) and contain even-

tually 1,000 or more new *Coxiella* cells. However, morphologically evident damage to these host cells is minimal, and persistent infections in which both host and parasite keep on multiplying have been maintained for several months (28, 30, 68). It is obvious that *C. burnetii* need not injure its hosts to reproduce.

As pointed out in the previous section, intact cells of *C. burnetii* utilize glutamate, glucose, and tricarboxylic acid cycle intermediates very slowly or not at all at pH 7 and very vigorously at pH 5 and below, almost certainly an adaptation of life in the phagolysosome (158, 159). ATP generated by the aerobic oxidation of glutamate is probably the chief source of energy for *C. burnetii*, although glucose and the tricarboxylic acid cycle acids may also serve as energy sources (158).

Although host-free multiplication has not been achieved, intact *C. burnetii* synthesizes DNA, RNA, and protein from appropriate monomeric intermediates at pH 4.5 (but not pH 7) and in the presence of glutamate. Parasites grown in either yolk sac (160) or baby hamster kidney cells (475) incorporate labeled glutamate or leucine into protein when incubated with the other naturally occurring amino acids in a high K^+-low Na^+ medium. Leucine incorporation continues for many hours, and the newly synthesized protein may amount to 15 or 30% of the total protein of the incubated *C. burnetii*. Polyacrylamide gel electrophoresis indicates that at least 24 different polypeptides are synthesized by host-free *C. burnetii*. Addition of exogenous ATP does not increase the rate of glutamate incorporation into protein (160). Some years ago it was found that pH 7 extracts of *C. burnetii* synthesize RNA from the four nucleoside triphosphates with phosphoenolpyruvate and pyruvate kinase as an energy-generating system (214). With the discovery of the acid-loving nature of the parasite, incorporation of labeled uridine into the cells of intact, host-free *C. burnetii* has been obtained at pH 4.5 and in the presence of glutamate (475). Little is known about the host-independent synthesis of DNA except that, again only at pH 4.5 and with glutamate as energy source, labeled thymidine is incorporated into parasite DNA (160). The level of phosphorylation at which *C. burnetii* captures host nucleosides for its own synthetic needs is not known. However, *C. burnetii* extracts make nucleoside triphosphates from the corresponding nucleoside mono- and diphosphates (84).

In host-free intact cells of *C. burnetii*, sustained synthesis of any one of the three major macromolecules is dependent on continued synthesis of the other two (475). Protein synthesis is immediately halted by rifampin (inhibits procaryotic RNA synthesis [138]), which confirms an earlier suggestion that *C. burnetii* messenger RNA is unstable (100), uracil incorporation into RNA is reduced by chloramphenicol (inhibits procaryotic protein synthesis [138]), probably because transcription enzymes are not synthesized, and inhibition of DNA synthesis by hydroxyurea (138) slowly reduces the rate of leucine incorporation into protein.

Rickettsiae. Soon after entry, rickettsiae escape from phagosomes (see section, "Survival") and multiply by binary fission (363) in the host-cell cytoplasm. Some cells of *R. rickettsii* (the agent of Rocky Mountain spotted fever) also multiply within host cell nuclei. The yield of infectious rickettsiae per host cell varies from about 100 for *R. rickettsii* to more than 1,000 for *R. prowazekii* and *R. tsutsugamushi*. The smaller yield of *R. rickettsii* is due to early release of rickettsiae from host cells (see below). Peak intracellular rickettsial populations are reached 3 to 5 days after infection (465, 466). Although most metabolic studies have used *R. prowazekii* (louse-borne typhus) or *R. typhi* (sometimes called *R. mooseri*), the agent of flea-borne typhus, the one major metabolic difference among the various rickettsial species appears to be that only *R. tsutsugamushi* (scrub typhus) does not require an atmosphere enriched in carbon dioxide for intracellular growth (226). High multiplicities of rickettsiae rapidly kill host cells by disrupting their plasma membranes (see section, "Entry"). At the lower multiplicities of infection used for metabolic experiments, *R. akari* (rickettsial pox) growing in L cells produces more visual evidence of host cell injury and inhibits synthesis of host DNA more severely than does *R. typhi* (452). Under the same conditions, *R. rickettsii* (376) is more damaging than *R. prowazekii* (377). It is not known if injury to host cells is essential for multiplication of rickettsiae, as it is for that of bdellovibrios. The observations that *R. tsutsugamushi* multiples in L cells only when they are suspended in a medium that supports active L-cell proliferation (196) and that vole cells infected with *R. rickettsii* multiply indefinitely in culture (406) strongly suggest that it is not. However, a protease-associated activity of *R. rickettsii* that is essential for parasite multiplication may result in host cell injury (434).

Rickettsiae obtain most of their metabolic energy by coupling the phosphorylation of ADP to ATP with the oxidation of glutamate via the tricarboxylic acid cycle, mainly to aspartate, carbon dioxide, and ammonia (47, 445, 456, 473). Glucose is not catabolized by either intact cells (310) or extracts (88). Exogenous ATP may fulfill the energy requirement for some rickettsial functions but not for others. Labeled amino acids are incorporated into rickettsial proteins with energy obtained from glutamate oxidation, but added ATP is needed for maximum rates of incorporation (50). Exogenous ATP inhibits the hemolysis of sheep erythrocytes (see section, "Entry") by undamaged rickettsiae (51), but it promotes their hemolysis by damaged rickettsial cells (51, 457). In a similar vein, active transport of lysine into rickettsial cells oxidizing glutamate is inhibited by both cyanide and dinitrophenol, but exogenous ATP reverses only the inhibition caused by cyanide and not that caused by dinitrophenol (381). Rickettsial activities in which oxidative phosphorylation accompanying glutamate oxidation cannot be replaced by supplying ATP from the outside are probably energized by a proton motive force (125) rather than by direct utilization of ATP (381, 473). Outside ATP is brought into the rickettsial cell by a membrane-located transport system in which a molecule of internal ADP is exchanged for a molecule of external ATP (458), whose energy is made available by the action of a membrane-bound adenosine triphosphatase (473).

Host-independent synthesis of rickettsial macromolecules has been demonstrated both with isolated rickettsial cells and with rickettsiae growing in host cells. Host-free rickettsiae exhibit a low rate of incorporation of labeled methionine (52) and glycine (50) into rickettsial protein. Maximum rates of incorporation require the presence of all naturally occurring amino acids, ATP, nicotinamide adenine dinucleotide, coenzyme A, a high K^+-low Na^+ medium, and the concomitant oxidation of glutamate. At an even lower rate, labeled acetate is incorporated into lipids (48). Rickettsial synthesis of proteins and nucleic acids in infected host cells may be demonstrated by using the antibiotic cycloheximide, which inhibits synthesis of protein and DNA in eucaryotes but not in procaryotes (138). In experiments patterned after those first done with chlamydiae (12, 13), the incorporation of labeled amino acids or adenine into trichloroacetic acid-insoluble fractions of nonmultiplying (irradiated) L cells in

which rickettsiae were growing was measured at several intervals after infection in the presence and absence of cyclohexamide (448, 452). Under these conditions, the cyclohexamide-resistant incorporation amounts to as much as 50% of the incorporation observed in its absence. For synthesis of nucleic acids, rickettsiae appear to rely on host-derived nucleoside monophosphates or to synthesize the monophosphates de novo. AMP is transported into rickettsial cells without hydrolysis (27, 454). Intact cells and extracts degrade ATP only as far as AMP (454), and host uridine and thymidine pools are not directly converted to the corresponding nucleoside phosphates (455).

Chlamydiae. Chlamydiae reproduce by binary fission (136, 186, 248) in membrane-bound vacuoles that do not fuse with host lysosomes (see section, "Survival"). Over a period of 2 to 3 days, the yield of new elementary bodies for each infecting one may vary from little more than 10 to almost 1,000 (290). Of the two chlamydial cell types (see section, "Entry"), the multiplying reticulate bodies are more active metabolically than the infectious elementary bodies. Most biochemical investigations have used *C. psittaci*, but there seems to be no major metabolic differences between the two chlamydial species (*C. psittaci* and *C. trachomatis*). Although cultured host cells are quickly killed by contact with large numbers of elementary bodies (see section, "Entry"), they may survive infection with one to five elementary bodies per host cell in good enough condition to undergo a limited number of divisions while at the same time adequately supporting chlamydial multiplication (44, 90, 197). However, persistent chlamydial infections in which both host and parasite multiply freely have not been obtained (see reference 291 for review). It seems most likely that host cell injury need not accompany chlamydial multiplication, but frequently does.

In adapting to the intracellular habitat, chlamydiae appear to have evolved mechanisms for exploiting the energy-rich compounds of their hosts and at the same time to have lost whatever energy-producing systems they might once have had (279). Host-free chlamydiae have neither flavoprotein nor cytochrome respiratory enzymes (E. Weiss and L. A. Kiesow, Bacteriol. Proc., p. 85, 1965), they catabolize glutamate, glucose, and pyruvate to a limited extent but without producing useful energy (444), and infected host cells do not develop novel energy-generating mechanisms (146, 281). However, host-free reticulate bodies of *C. psittaci* move ATP in and ADP out of their intracellular space with a translocase similar to that of rickettsiae, hydrolyze it to ADP with a chlamydial adenosine triphosphatase, and use ATP energy for macromolecular synthesis (178, 179).

As with rickettsiae, host-independent synthesis of chlamydial macromolecules has been shown in both isolated chlamydiae and chlamydiae-infected host cells. In a high K^+-low Na^+ medium and in the presence of ATP and 19 unlabeled amino acids, *C. psittaci* reticulate bodies incorporate labeled methionine into many proteins with molecular weights identical to those of chlamydial proteins made in infected L cells (178). The incorporation is inhibited by chloramphenicol but not by cycloheximide. In the presence of all four ribonucleoside triphosphates, reticulate bodies of *C. psittaci* incorporate labeled guanosine triphosphate into RNA (397), and elementary bodies of *C. trachomatis* make labeled RNA from labeled uridine triphosphate (360). Synthesis of DNA in isolated chlamydiae has not been reported. Host-independent synthesis of chlamydial macromolecules has been demonstrated with the cycloheximide technique already described for rickettsiae. In the presence of enough cycloheximide to inhibit more than 90% of the protein synthesis by L cells, *C. psittaci* incorporates labeled amino acids into chlamydial protein (12, 13). Incorporation is maintained at a high level as long as the L cells are viable, and it is inhibited by chloramphenicol and tetracycline (inhibits procaryotic protein synthesis [138]). Comparable experiments (40) have been done with *C. trachomatis* and FL cells (derived from human amnion) treated with emetine, another inhibitor of eucaryotic protein synthesis (138). The ability of cyclohexamide to block L-cell synthesis of DNA, as well as that of protein, allows the demonstration of host-independent incorporation of uridine into chlamydial DNA (13). Emetine, which also inhibits RNA synthesis in FL cells (145), has been used to show host-independent synthesis of RNA by *C. trachomatis* (156).

Chlamydiae readily incorporate most medium-supplied nucleosides into DNA and RNA, but the pyrimidine deoxyribonucleosides are utilized very poorly (416). *C. psittaci* has no detectable thymidine kinase of its own, and it grows normally in thymidine kinase-deficient L cells (177, 247). Chlamydiae probably make the most of the thymidine triphosphate needed for DNA synthesis from uridine via thymidylate synthetase. *C. psittaci* uses guanine as a nucleic acid precursor but only in host cells with a hypoxanthine guanine phosphoribosyl transferase to elevate guanine to the nucleotide level (76). The utilization of nucleoside triphosphates as nucleic acid precursors by host-free chlamydiae has already been described. An equilibrium-labeling technique has been used to show the same pattern of utilization in infected host cells (175). Exogenous uridine and adenine are incorporated into *C. psittaci* RNA at rates consistent with the chlamydiae drawing exclusively on the nucleoside triphosphate pools of the L-cell hosts.

As in *C. burnetii*, synthesis of chlamydial DNA, RNA, and protein is interdependent. Chloramphenicol inhibits RNA synthesis by *C. trachomatis* in FL cells (156), and with *C. psittaci* multiplying in L cells, addition of chloramphenicol, rifampin, or nalidixic acid (inhibits procaryotic DNA synthesis [138]) brings the synthesis of all three macromolecule classes to a halt within a few hours (415).

Malarial parasites. Studies on the biochemistry of plasmodial multiplication have, like those on entry, been almost entirely restricted to the blood stages of these parasites (reference 428 reviews some pioneering studies on the biochemistry of exoerythrocytic stages). An erythrocytic merozoite enters a new erythrocyte contained in a parasitophorous vacuole derived from the erythrocyte membrane (see section, "Entry"), and, always within the confines of an expanding parasitophorous vacuole, it grows and multiplies by a process called schizogony (141). Over a period of 1 to 3 days, the small uninuclear merozoite grows into a large schizont with 6 to 20 nuclei, which segments into a new brood of merozoites that are released into the bloodstream when the parasitized erythrocyte disintegrates. Most metabolic studies have used *P. falciparum* cultivated in vitro in human erythrocytes (412, 414), *P. knowlesi* grown in monkeys, *P. berghei* grown in rats and mice, *P. gallinaceum* grown in chickens, and *P. lophurae* grown in ducks. There are significant metabolic differences among these parasites, particularly between the mammalian and avian malarias. The metabolic activities of plasmodia have been observed inside the erythrocyte (parasitized erythrocytes) or outside it (free parasites). Both kinds of parasite preparation may be contaminated with metabolically active host material such as immature erythrocytes, leukocytes, platelets, erythrocyte membranes, and vacuolar membranes which still enclose the

not-so-free parasite. The extent of this contamination was not fully realized by early investigators (myself included) whose work must, therefore, be interpreted with caution. Even recent investigations are not always without ambiguity. In consequence, many an important question about plasmodial metabolism is still begging an answer.

Of the intracellular parasites of Table 2, only the erythrocytic stages of malarial parasites live in nondividing host cells to which the classic test of host cell damage, inhibition of multiplication, cannot be applied. However, growing malarial parasites change and consume their erythrocyte hosts to an extent exceeded only by the bdellovibrios. The surface of the parasitized erythrocyte is altered both structurally and functionally (3, 367, 413), presumably to make a better habitat for the parasite, and its hemoglobin is extensively degraded (discussed below).

One of the few undisputed facts of plasmodial metabolism is that glucose is the preferred energy source for every species examined (193, 194, 367). Erythrocytes parasitized with *P. berghei* and *P. lophurae* actually catabolize glucose at rates exceeding its rate of entry into unparasitized host cells. The plasmodia have solved this dilemma by bringing about an increase in the rate at which glucose enters the parasitized erythrocyte (299, 371). The related blood-dwelling *Babesia rodhaini* brings about a similar change in its mouse erythrocyte host (195). A second undisputed fact is that malarial parasites catabolize glucose mainly to lactic acid (193, 194, 367). ATP is probably synthesized by substrate-level phosphorylation, but this has not been demonstrated directly. Beyond this, little is known for sure. Mammalian malarias produce little else but lactic acid from glucose, whereas avian malarias also generate organic acids and carbon dioxide, possibly by the citric acid cycle or other as yet undefined aerobic routes. However, it is even argued whether or not malarial parasites actually consume any oxygen at all (cf. references 194 and 367).

It is not easy to demonstrate an unequivocal host-independent synthesis of protein by a eucaryotic parasite located inside a eucaryotic host (selective inhibitors are not available), but there is no reason to believe that the protein-synthesizing machinery of the host is needed to make malarial protein (194, 366, 367). In parasitized erythrocytes, amino acids for synthesis of parasite protein come from either the outside (plasma) or the inside (hemoglobin). *P. knowlesi* needs medium-supplied methionine and leucine for growth in monkey erythrocytes, and it concentrates these amino acids (and others as well) from the medium (137, 266, 267, 327). Nevertheless, 80% of the methionine in *P. knowlesi* protein still comes from hemoglobin (137), which is probably the most important source of amino acids for malarial parasites. Hemoglobin is taken into the ingestive organelle of the parasite (4, 357) where it dissociates into globin and malarial pigment, an iron-porphyrin complex of disputed structure (194, 367). The globin is then hydrolyzed to free amino acids by a plasmodial protease(s) (372). That the globin-derived amino acids are incorporated into malarial protein is shown by labeling the hemoglobin before infecting the erythrocytes (137, 400).

Plasmodia make nucleic acids independently of their hosts. For example, mouse erythrocytes (which synthesize no nucleic acids) infected with *P. berghei* incorporate ^{32}P into DNA and RNA (453), duck erythrocytes (which have nuclei and make nucleic acids) parasitized with *P. lophurae* incorporate ^{32}P into parasite DNA without its passing through intermediates of the host cell nucleus (437), and *P. gallinaceum* freed from chicken erythrocytes continues to make DNA (85). Free parasites of *P. lophurae* incorporate labeled orotic acid (410) and adenosine (369) into nucleic acids, as do monkey erythrocytes containing *P. knowlesi* (368). Malarial parasites do not make their own purines and must obtain them from outside sources as free bases or as nucleosides, whereas they synthesize pyrimidines de novo and, with the exception of orotic acid, do not incorporate exogenous pyrimidine bases or nucleosides into malarial nucleic acids (194, 225, 367). A possible role for exogenous ATP in plasmodial metabolism is suggested by the requirement of ATP for the survival of free parasites of *P. lophurae* in an in vitro system that supports development of multinucleate forms from uninucleated ones but does not permit net multiplication (408, 410, 477) and by the depletion of ATP stores in erythrocytes infected with mammalian (57) and avian (409) malarias.

General Aspects of the Multiplication of Intracellular Parasites

Exploitation of host activities. All organisms grow and multiply by dissimilating food to yield energy and synthetic intermediates and from them synthesizing DNA, RNA, protein, and other cell constituents. If we believe in the unity of biochemistry, then we must also believe that these processes are basically the same in the intracellular parasite and in its host cell. Therefore, there are three broad areas of host cell activity, any or all of which the enterprising intracellular parasite may divert to its own ends. They are synthesis of intermediates, generation of energy, and synthesis of macromolecules.

Of all of the intracellular parasites, only the viruses are supposed to exploit host cell macromolecules. However, *P. berghei* grown in mouse erythrocytes contains mouse superoxide dismutase, whereas *P. berghei* from rat erythrocytes has the corresponding rat enzyme (118, 119). If it turns out that these host enzymes are really functionally associated with *P. berghei* and not just adventitiously absorbed, then the possibility of other host enzymes being appropriated by other parasites must be considered. The transport of proteins into eucaryotic cells by endocytosis is well known (317, 378); the situation with procaryotic cells is less satisfactory.

So far, only the chlamydiae appear to be totally dependent on their host cells for ATP and other energy-rich molecules (178, 179). However, partial dependency has been demonstrated in rickettsiae (50–52) and suspected in malarial parasites (408, 410, 411) and microsporidia (443). Utilization of exogenous ATP requires the presence in an intracellular parasite of a membrane-located ATP-ADP transport system, which has so far been demonstrated only in rickettsiae (458) and chlamydiae (179). The occurrence of this enzyme system, found otherwise only in mitochondria (460), in intracellular parasites of only the most distant phylogenetic kinship (290) is a fine example of convergent evolution at the molecular level. In transporting and directly utilizing host-derived nucleoside monophosphates, bdellovibrios (348) and rickettsias (27, 454, 456) are exhibiting a parallel but less energy-efficient adaptation. On the basis of an admittedly small number of species examined, it appears that one general adaptation to the intracellular habitat is the exploitation of the energy stored in the phosphate bonds of the nucleic acids and nucleoside phosphates of the host cell.

There seems to be no grand pattern in the choice of energy source by intracellular parasites. It is true that *Bdellovibrio* (185), *Coxiella* (158, 159), and *Rickettsia* (53) preferentially utilize glutamate, but this amino acid is also the favorite

substrate of many free-living saprophytic bacteria (233). What possible sense is to be made of the observations that *Plasmodium* catabolizes glucose but not glutamate, that *Rickettsia* does just the opposite, and that *Coxiella* uses them both?

It is a truism that the host cell supplies intermediates to the intracellular parasite. That this supplier-consumer relation is a peculiar one is less widely appreciated. The host cell may be at the same time the environment in which the parasite lives, the source of synthetic intermediates, and a competitor with the parasite for some of these same intermediates (176, 280, 282, 283). Competition has been most studied in *C. psittaci* and L cells. Chlamydiae and host cells sometimes compete on an even footing, as when *C. psittaci* incorporates uridine into its rRNA at a rate consistent with free access to the ribonucleoside triphosphate pools of the L cell (175), but it completes poorly with the L cell for thymidine (177). Only in the presence of exceptionally high external levels of the nucleoside, and with the host cell synthesizing the deoxyribonucleoside phosphates of thymine, do the chlamydiae incorporate even traces of thymidine into their DNA. A more complicated case of host-parasite competition is provided by L-isoleucine, which neither host nor parasite can synthesize (176). L cells ingest *C. psittaci* in the absence of this amino acid, but neither host nor parasite divides until isoleucine is added. The denser the infected L-cell population, the higher the concentration of amino acid needed to set both L cells and *C. psittaci* to multiplying, and at no isoleucine level does one start dividing before the other. When cycloheximide is added to block incorporation of amino acids into L-cell protein, *C. psittaci* grows in isoleucine-deficient media on the small amount of the amino acid liberated by the breakdown of host protein, which shows that it is not the absolute concentration of isoleucine that controls initiation of chlamydial multiplication but the level at which the parasite is able to compete effectively with its host.

There is no reason to believe that competition between host and parasites is unique to chlamydiae, although data are scarce. *R. typhi* (455), *Toxoplasma gondii* (321), and *P. berghei* (63) also have difficulty in incorporating thymidine into DNA, probably because, as with chlamydiae, they cannot phosphorylate the nucleoside and cannot compete effectively for host-made thymine deoxynucleoside phosphates. Conversely, *Toxoplasma gondii* growing in human diploid fibroblasts incorporates virtually all added uracil into its own DNA because it has an active uridine phosphorylase and its host cell does not (324). It is probably no coincidence that the two parasites of Table 2 which make use of host macromolecules as a major source of synthetic intermediates, the bdellovibrios and the malarial parasites, are the only ones that do not have to compete with host cells that are also trying to multiply.

An interesting example of competition between two different intracellular parasites is the exclusion of *R. rickettsii* from its tick host *Dermacentor andersoni* by nonpathogenic rickettsiae, a competition that may determine the geographic range of *R. rickettsii* (64). This interspecific competition probably occurs at the level of the host cell, not the entire host.

Modification of host cell membranes. Of the parasites of Table 2, only the rickettsiae multiply in the main intracellular space of the host cell (see section, "Survival"). The others are all segregated in spaces enclosed by membranes of host origin. The space and the membrane are called different things in different host-parasite associations; I will call them the vacuole and the vacuole membrane. In bdellovibrio-host bacterium interactions, the intraperiplasmic pocket, which is the functional equivalent of the cytoplasmic vacuole in eucaryotic hosts, has an enclosing membrane whose function is to keep host-derived nutrients from diffusing beyond the reach of the growing parasite, but in *Coxiella*, *Chlamydia*, and *Plasmodium* infections, the vacuole membrane presents an additional barrier through which all materials for parasite growth must pass before actually encountering the parasite surface. Because the vacuole membrane increases perhaps 10- to 100-fold in mass and surface area as the parasites grow and divide, it has occurred to many that, as the membrane is enlarged, it may be modified by the parasite brood it encloses in such a way as to better provide for the growth needs of those parasites.

That the vacuole membrane can be different from the plasma membrane of the host cell from which it originates (see section, "Entry") is well established. However, exactly what the differences are, how the parasite brings them about, and how they contribute to parasite multiplication are seldom obvious. The vacuole membrane of *P. lophurae* tightly surrounds the parasite when it is artificially liberated from its host cells, and when the parasite grows extracellularly, the vacuole membrane grows proportionally and participates in the uptake of food by the ingestive vacuole (235, 237). In vacuole membranes, the number and distribution of membrane proteins are not those expected of erythrocyte membranes (236, 268, 370). Evidence for modification of other vacuole membranes is more indirect. For example, *C. psittaci* appears to modify its vacuole membrane so as to prevent fusion with lysosomes (see section, "Survival"), and it must be assumed that, if this parasite is to use host nucleoside triphosphates, they must be transported through a modified vacuole membrane as well as the chlamydial membrane (286).

Intracellular parasites also change the outer membranes of their host cells, usually in ways that appear to be beneficial to the parasites. By deacetylating peptidoglycan, bdellovibrios render their host immune to further infection (402). In a comparable fashion, L cells persistently infected with *C. psittaci* are also immune to superinfection because their surface proteins have been altered to make them incapable of associating with exogenous chlamydiae (292, 293). Malarial parasites bring about a number of changes in the surface properties of infected erythrocytes (reviewed in reference 413). In some species, permeability to glucose is increased (299, 371). Schüffner's dots, long known to appear on erythrocytes infected with *P. vivax*, have been shown by electron microscopy to be small invaginations in the erythrocyte plasma membrane and are thought to be involved in pinocytosis (3). *P. falciparum*-infected erythrocytes have knoblike protrusions which cause them to stick to the capillary endothelium, thus bringing about the sequestration of mature-stage parasites in the capillary bed that is characteristic of falciparum malaria (252, 421). Some workers have concluded that the knobs contain *P. falciparum* antigens (224, 301), one of which is reported to be homologous with the histidine-rich protein implicated in entry of *P. lophurae* (222, 223), whereas others have suggested that these antigens represent newly exposed or chemically modified erythrocyte components (155).

Regulation of onset and cessation of intracellular multiplication. How does the intracellular parasite know when it has reached an intracellular site that will support growth and multiplication? How does it avoid abortive turn-on? A general indication that intracellular parasites have evolved

suitable control mechanisms is the frequency with which they undergo morphological transformation on entering host cells; that is, there are morphologically distinguishable extracellular transit forms and intracellular reproductive forms (see section, "Transit"). Of the five intracellular parasites described in this section, only the rickettsiae do not conform to this pattern (Table 2).

The intracellular parasites of Table 2, other than *Bdellovibrio*, live in vertebrate host cells in which the major intracellular cation is K^+ and the predominant cation in extracellular fluids is Na^+. Because extracellular development of *P. lophurae* (408) and synthesis of macromolecules by host-free *Coxiella burnetii* (475), *R. prowazekii* (50, 52), and *C. psittaci* (178) proceed optimally in high K^+-low Na^+ media, it is possible that a high concentration of K^+ favors onset of intracellular multiplication (perhaps in concert with parasite-specific factors) or that high Na^+ levels inhibit the initiation of multiplication. In a comparable way, the facultative intracellular bacterium *Y. pestis* (the plague bacillus) and the closely related *Y. pseudotuberculosis* respond to change from a medium high in Na^+ and Ca^{2+} to one high in K^+ and Mg^{2+} by shifting from extracellular to intracellular patterns of metabolism and growth (59–61).

Infectious, flagellated bdellovibrios do not make DNA (338, 356), and although structural modification of host membranes begins as early as 20 min after entry into the bacterial host, DNA synthesis and filamentous growth do not start for another 40 min (259). These observations have been interpreted to mean that bdellovibrios must be in the intraperiplasmic space for about 1 h before they become responsive to a signal for starting DNA synthesis that is received only in that habitat (346, 355).

The dependency of both catabolic and anabolic processes in host-free *Coxiella burnetii* on the low pH of the intraphagolysosomal environment has already been described. This dependency appears to be a real mechanism for limiting the metabolic activities of the parasite to the phagolysosome (and not just a coincidence). Raising the intraphagolysosomal pH of infected cells inhibits the multiplication of *Coxiella burnetii* (158), and intracellular procaryotes (*Rickettsia* and *Chlamydia*) that do not live in phagolysosomes optimally catabolize the common substrate glutamate at pH 7, not pH 4.5 (160). Amastigotes of *L. mexicana*, which also live in phagolysosomes, optimally catabolize glucose, glutamate, and other substrates at neutral, not acid, pH (167), but with glucose as energy source they incorporate more leucine into protein at pH 4.75 than at pH 7.4 (K.-P. Chang, personal communication). A developmental cycle for *Coxiella burnetii* has been proposed in which an endospore-like cell type alternates with a vegetative cell type (263). If continued investigation substantiates this suggestion, then the stimulus for transition between the endospore-like form and the vegetative form may prove to be the low pH of the phagolysosome.

As already stated, rickettsiae either generate their own ATP by oxidative phosphorylation of glutamate via the tricarboxylic acid cycle (47) or take up ATP from the host cytoplasm by means of an ATP-ADP transport system (458). The relative contribution of each potential source of ATP to rickettsial energy needs is probably decided by adenylate-dependent (ATP > ADP > AMP) regulation of a key enzyme in the tricarboxylic acid cycle, citrate synthetase (325, 459). Extracellularly, the rickettsiae need ATP for getting inside host cells (see section, "Entry"), the adenylate concentration outside the host is low, citrate synthetase is active, and ATP is produced via the tricarboxylic acid cycle. Intracellularly, citrate synthetase is inhibited by the high adenylate levels, and the rickettsiae turn to the host cell for their ATP.

In *Chlamydia*, the signal for initiation of multiplication appears to be provided by host-generated reductants in the intracellular environment. Unlike most procaryotes, chlamydiae have no peptidoglycan layer in their cell envelopes (35, 142), and the rigidity of the elementary body wall resides in large disulfide bond-linked complexes of the major outer membrane protein (37, 178, 182, 302, 398). When elementary bodies enter host cells, the cross-linked complexes are reduced to the monomeric state (T. P. Hatch and G. Hood, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K13, p. 149). Three lines of evidence suggest that this reduction increases the permeability of the elementary body envelopes to ATP and other nucleoside triphosphates which they need for reorganization into multiplying reticulate bodies. They are as follows: (i) host-free reticulate bodies, but not elementary bodies, transport ATP and other nucleoside triphosphates (178, 179); (ii) isolated elementary bodies synthesize RNA from exogenous nucleoside triphosphates, but only after they have been treated with the reducing agent mercaptoethanol (397); and (iii) studies with liposomes made from chlamydial outer membrane complexes suggest that intact outer membanes have pores large enough to accommodate nucleoside triphosphates, but only when care is taken to avoid the formation of disulfide bond-linked complexes (37).

After the malarial merozoite penetrates the host erythrocyte, it discards the organelles associated with entry and differentiates into a uninuclear trophozoite which grows into a mature schizont with many nuclei. Such drastic morphological changes must be the result of complex metabolic activity set in motion by a stimulus received in the intraerythrocytic environment. Merozoites of *P. lophurae* developing extracellularly (237) must receive and respond to a similar stimulus, but no one knows what it is.

After a period of multiplication that varies from hours to days in different host-parasite systems, the end of the reproductive phase is often heralded by the differentiation of some multiplying forms into infectious transit forms, although some parasites, such as chlamydiae (136, 187, 248), continue to produce both cell types for many hours. The morphological aspects of this differentiation have been well described for plasmodia (3) and chlamydiae (136, 187, 248), but only for the bdellovibrios is there any hint of underlying mechanisms. When filamentous, growing bdellovibrios are prematurely released from host bacteria, they complete one round of DNA replication and then fragment into infectious forms in the absence of external sources of carbon for energy or of any special stimulating factor (356). It appears that, for the prematurely released bdellovibrios, the signal for cessation of multiplication and onset of differentiation is removal from their normal habitat and that, for the bdellovibrios left undisturbed in the intraperiplasmic space, the corresponding signal is exhaustion of a regulatory component (356). Exhaustion of a metabolite or inactivation of a regulator is also the best guess as to what controls differentiation of schizonts into merozoites and reticulate bodies into elementary bodies.

Synthesis of unique parasite constituents. In attempting a comparative treatment of the metabolic processes underlying multiplication of intracellular parasites, I have ignored a significant body of information on how each of these organisms synthesizes many substances unique to its particular species. Some, such as the histidine-rich protein of malarial parasites (220, 221) and the glycanase of bdellovibrios (401),

may have appeared as positive adaptations to intracellular life. Others, such as the folates (87, 123) and lipids (192, 256, 337) made by chlamydiae and malarial parasites, were probably already being synthesized by the free-living ancestors of these organisms. In the path toward adaptation to the intracellular habitat, there appears to have been no drift towards loss of individuality.

PRESERVATION OF HOST FUNCTION

Predators kill their prey and eat them on the spot, whereas parasites only slowly consume their living hosts. Hosts are either not killed at all or only after reproduction of the parasites and dissemination of their progeny are assured (382). A good illustration of this principle is the history of myxomatosis in Australia (121). In the middle of the 19th century, wild European rabbits were introduced into that continent, whereupon they soon became devastating pests. One hundred years later, myxoma virus, which causes a nearly 100% lethal infection in European rabbits, was deliberately liberated as a potential agent of biological control. Enormous numbers of rabbits were killed, but in the course of a few years the original highly lethal virus was replaced by viruses of lesser lethality that were more efficiently transmitted by their mosquito vectors and were, therefore, more likely to survive.

Preservation of Function in Acute Infection of Host Cells in Culture

Of the five intracellular parasites of Table 2, only the bdellovibrios are predacious; that is, only they kill their hosts (= prey?) and consume them. Malarial parasites partially consume their erythrocyte hosts, but they depend on continued erythrocyte function to bring them nutrients from the outside. The other three, *Rickettsia*, *Coxiella*, and *Chlamydia*, injure host cells to varying degrees depending on the nature of the host cell, the particular species and strain of parasite, and the multiplicity of infection. However, for each of these three infectious agents, there is at least one set of conditions under which parasite and infected host cell multiply simultaneously. This means that these parasites need not impair host cell function to multiply, but it does not necessarily mean that they have evolved ways to prevent it.

At a constant multiplicity of infection, the rate at which an intracellular parasite multiplies is probably the most important determinant of host cell injury and thus also of host cell preservation. If intracellular parasites have indeed evolved ways of regulating their reproductive rates, a likely mechanism is one in which the parasite responds to changes in the concentration of the constituents of the small molecule pools of the host, changes which may at least in part be brought about by parasite activity (176, 278, 459). For example, in mouse fibroblasts (McCoy cells) treated with cycloheximide to suppress host-parasite competition, the rate of *C. psittaci* multiplication may be varied by changing the concentration of leucine, valine, or phenylalanine (16).

Preservation of Function in Persistent Infection of Host Cells in Culture

Persistent infections in which host and parasite coexist for a long time, sometimes for the lifetime of the host, are perfect examples of how parasites do not destroy host functions needed for their continued existence. Although persistent infections may be equally common in diseases caused by either viral or organismal intracellular parasites, cell culture models of persistent infection have been studied much more intensively with viruses (135, 255). The organismal infectious agents have followed an evolutionary course parallel to the one pursued by the viruses, so that, as with viruses, two kinds of persistently infected host cell populations may be recognized (121). In carrier cultures, the parasite infects and multiplies in only a relatively small fraction of the host cell population. The persistence of the parasite is assured, and all of the host cells are never infected and destroyed. The persistent infection of McCoy cells with a trachoma biovar of *C. trachomatis* is an example of a carrier culture (241, 242). However, it is the other kind of persistent infection, the steady-state culture, that best illustrates maintenance of host function in the presence of a multiplying population of intracellular parasites. In these cultures, all or nearly all of the host cells are infected, large amounts of infectious agent are produced, and multiplication of the host cell population is only minimally interfered with.

Coxiella burnetii. That *C. burnetii* persistently infects humans has been shown by isolating the organism from the aortic valves in fatal cases of Q fever endocarditis and from the placentas of women who had Q fever as long as 3 years before childbirth (29). Therefore, it is not surprising that *C. burnetii* readily establishes persistent infections in a variety of cell lines (28, 30, 68). No matter what the initial multiplicity of infection, all of the host cells become infected in a few days. Both *C. burnetii* and the host cells keep on dividing, and the infected cultures persist for months. How host cell and parasite accommodate each other is not known. In comparing persistent infection of L cells and Vero cells, it was noted that the lysosomal response diminished with time in L cells but that there was no such diminution in Vero cells (28).

R. rickettsii. With the exception of Brill-Zinsser disease, a recrudescence of louse-borne typhus years after the original infection (383), persistent rickettsial infections do not seem to occur in humans, although such infections have been postulated to occur in nonhuman vertebrate hosts of rickettsiae. Early in the study of rickettsial growth in cell culture, it was observed that mouse lymphoblasts grow and divide for several days after infection with *R. tsutsugamushi* (54), but long-term persistent infections have been described only for *R. rickettsii* grown in a cell line derived from the tunica vaginalis of voles (406). All of the host cells become infected, but the only structural alterations are swelling of the rough endoplasmic reticulum and formation of electron-translucent vacuoles. In contrast, when Vero cells are infected under identical conditions, they are all killed.

C. psittaci. Persistent infection of birds and mammals (including humans) frequently occurs in diseases caused by *C. psittaci* (362). When L-cell monolayers are infected with even the lowest multiplicities of *C. psittaci*, the monolayers appear to be completely destroyed in a few days by successive rounds of chlamydial multiplication and spread to previously uninfected cells. However, in 2 to 4 weeks, a few colonies of L cells emerge from the ruins of the infected monolayers. All L cells in these colonies are infected with *C. psittaci*, and L-cell populations derived from one of these colonies may be maintained indefinitely in a persistently infected state (287, 291–293). Survival of both L cells and chlamydiae is achieved by alternation of periods of host cell multiplication during which the chlamydiae also multiply, but in an unusual covert manner that does not destroy the host cell or materially slow its rate of division, with periods of overt chlamydial multiplication in which large numbers of infectious chlamydiae are released, and most of the L cells are destroyed. In covertly infected L cells, chlamydiae are

not visualized by conventional staining, and the only signs of infection are resistance to superinfection with exogenous chlamydiae and alterations in cell surface proteins (292, 293). The shift from covert to overt mode of multiplication is accelerated in cultures growing at high L-cell density and slowed in cultures growing in a rich medium (287). Perhaps *C. psittaci* is too easily transmitted and too destructive to establish carrier-type persistent infections or steady-state infections such as those described for *Coxiella burnetii* and *R. rickettsii*. Perhaps a specific adaptation, the ability to shift to a covert, nondestructive multiplying form, had to be made before *C. psittaci* could set up persistent infections. Persistent infections with *C. psittaci* are functionally analogous to persistent virus infections in which the viral genome is integrated into the genome of its host, even though no such integration has occurred. This may be another example of convergent evolution in the common intracellular environment.

***Chlorella* and *H. viridis*.** The striking resemblance between the establishment of a symbiotic relationship between the freshwater coelenterate *H. viridis* and algae of the genus *Chlorella* has already been noted (see sections, "Entry" and "Survival"). This relation also has many of the characteristics of a steady-state persistent infection. After the algae are taken into host cells in individual vacuoles that do not provoke lysosomal fusion, their cell division is brought into harmony with that of their hosts so that the association persists without multiplication of one member of the symbiotic pair outstripping that of the other (294). The number of algae per hydra varies with the need of the nonphotosynthetic host for the help of its photosynthetic partners. In starved and illuminated hydra, there may be as many as 25 algae in each host cell, but when *H. viridis* is fed and kept in the dark, the mechanism preventing phagosome-lysosome fusion is overridden, and all but as few as 3 algae per hydra are either digested intracellularly or expelled intact (300). The principal factor regulating *Chlorella* multiplication in starved *H. viridis* may be the concentration of sulfate ion, which is essential for division of algal cells. Raising the concentration of sulfate ions doubles the number of algae per host cell. There is also the possibility that dividing host cells stimulate algal division or that nondividing host cells inhibit it (261).

Do Intracellular Parasites Really Preserve Host Cell Function?

There is no doubt that, under certain conditions, a number of host cells and their intracellular associates adjust to each other in ways that allow long-sustained multiplication of each. The doubt is about how this adjustment is brought about. Students of parasitism like to think of these adjustments as adaptations on the part of the parasite, adaptations that preserve its intracellular habitat. On the other hand, students of symbiosis like to think of what appear to be very similar adjustments as host adaptations that regulate symbiont multiplication to the benefit of the host. In both kinds of sustained two-party associations, the only kind of regulatory factor established with any degree of plausibility is the intracellular concentration of metabolites needed by the parasite (= symbiont), and what controls these concentrations is not known. Yet another explanation might be that the multiplicity of infection of host cells in intact natural hosts is normally so low that impairment of host cell function is never a problem and that there has consequently been no selection pressure on intracellular parasites to come up with adaptations that favor maintenance of host cell functions.

RELEASE

If an intracellular parasite is to survive as a species, each new generation of infectious progeny must find its way into new host cells that provide environments suitable for multiplication and continued dissemination of the parasite. Although the concepts of K- and *r*-strategies (254, 387) were not formulated with single-celled, asexually reproducing organisms in mind, intracellular parasites are indubitably *r*-strategists. They are very small, and they colonize discontinuously distributed, unstable habitats (286). Migration occurs at every generation, and it is extremely wasteful. K-strategists are, in contrast, large, long-lived, and with a big investment in each offspring. Conventional *r*-strategists counteract loss to predators by producing large numbers of offspring, and intracellular parasites similarly counteract the failure of most released progeny ever to find suitable host cells. "Migration" of intracellular parasites from one host cell to another will be treated under the separate headings of "Release" and "Transit," although it is seldom clear where the one ends and the other begins.

Several different patterns of release may be visualized. After extensive parasite multiplication, infected cells may suddenly burst open and release a new brood of parasites, or intact host cells may continuously shed infectious progeny over a considerable fraction of the entire period of parasite multiplication. Infected cells may break open because of intolerable mechanical or osmotic stress resulting from unrestricted parasite multiplication, or they may lyse because the parasite secretes a lytic enzyme at one particular stage in its growth cycle. In a similar fashion, continuous parasite release may represent a parasite-directed activity, or it may be the adventitious result of normal membrane fluxes.

Patterns of Release

Release has not been a favorite subject among students of intracellular parasitism, but enough has been learned to suggest that several, possibly all, of these hypothetical patterns of release have actually been evolved.

***B. bacteriovorus*.** Bdellovibrios provide a simple model for release, just as they did for multiplication. Breakup of the filamentous multiplying form into fragments is the signal for induction of an enzyme(s) that lyses the bdelloplast by solubilizing the amino sugars and diaminopimelic acid of the deactylated host peptidoglycan (401–403). This enzyme lyses only the bdelloplast in which it was synthesized. Lytic enzyme released into the medium does not attack unlysed bdelloplasts, probably because it does not penetrate their outer membranes.

Rickettsiae. The release of rickettsiae from host cells furnishes a rare example of divergence among species that almost certainly share a comparatively recent evolutionary ancestor (295).

Secondary chicken embryo fibroblasts infected with *R. prowazekii* are packed with rickettsiae 72 to 96 h after infection, at which time some of the cells suddenly break up. Release continues for many hours. Electron microscopic studies failed to elucidate the events leading to host cell lysis. Cytopathic changes are only apparent late in the infection, when the host cells have already begun to disintegrate (377). Bursting might be due to overburdening the host cells with large numbers of rickettsiae or it might result from the action of phospholipase A (461, 462), which has

been firmly implicated in the entry of *R. prowazekii* into host cells (see section, "Entry").

The interaction of chicken embryo fibroblasts or L cells with *R. rickettsii* is initially much like infection with *R. prowazekii*, but as early as 10 h after infection intact host cells begin to release infectious rickettsiae (465). Rickettsiae fail to accumulate in host cell cytoplasm, there is a substantial buildup of rickettsiae in the growth medium, and there is a rapid spread of infection to initially uninfected host cells. Again, electron microscopic examination did not define the mechanism of release from intact cells. There is a loss of plasma membrane integrity, but only late in infection after extensive shedding of *R. rickettsii* has already occurred (376). Since phospholipase A also appears to play a role in the entry of *R. rickettsii* (432), it may also be involved in release, although a mechanism for participation of this enzyme in the release of either rickettsial species has not been proposed. Early release of *R. rickettsii* from host cells may be responsible for the rapid spread of infection characteristic of spotted fever.

R. tsutsugamushi offers what is probably yet a third pattern of rickettsial release, although it does resemble that of *R. rickettsii*. As first seen by light microscopy (363) and later by electron microscopy (117, 186), *R. tsutsugamushi* is extruded in projections from the surface of intact cells. In peritoneal mesothelial cells from infected mice, rickettsiae are seen at the free cell surface within evaginations of the plasma membrane, sometimes connected by a stalk to the host cell (117). They are released still surrounded by host cell membranes. These membrane-bound rickettsiae infect other host cells, but rickettsiae without membranes do not. Extracellular *R. rickettsii* is also sometimes surrounded by host membranes, but only late in infection when most host cells have already been infected (376).

C. psittaci. When *C. psittaci* grows in L cells, intracellular elementary bodies (the infectious chlamydial cell type) are first seen 15 to 20 h after infection (136, 187, 248). At the same time, extracellular infectivity begins to rise, and the number of trypan blue-staining L cells (a measure of damage to the plasma membrane [217]) increases. This early appearance of extracellular infectivity could be due to either shedding from intact cells or disintegration of a few early damaged host cells. However, the great bulk of *C. psittaci* infectivity is released 20 to 40 h after infection, when most infected cells are dying and breaking up. Lysosomal enzymes released late in infection have been associated with host disintegration and lysis (407). A protease made by *C. psittaci* in L cells may also facilitate release (392). An electron microscopic study of the shedding of the polyarthritis strain of *C. psittaci* into the intestinal lumen of orally infected calves illustrates the potential complexity of chlamydial release in intact hosts (101). Chlamydiae are released in three ways: (i) infected cells rupture and release their contents, (ii) entire infected cells are extruded into the intestinal lumen, and (iii) small groups of chlamydiae are released enclosed in cytoplasmic fragments.

Malarial parasites. Merozoites of *P. knowlesi* are released with sudden explosiveness from monkey erythrocytes containing mature schizonts (103). After the erythrocyte membrane vesiculates (the first visible sign of impending release), the erythrocyte swells and bursts within a minute. Merozoites appear to leave erythrocytes in the same orientation in which they entered, apical complex first, which has inspired the suggestion that the mechanisms of entry and release may be the same (206). It has also been suggested that the accumulation of osmotically active substances in the parasite vacuole may cause the rupture of host cells (32) or that plasmodial proteases may take part in lysis of the erythrocyte membrane (372).

General Aspects of Release

Incomplete release. In cell cultures and in chicken embryos, release of intracellular parasites from host cells is frequently incomplete. When attempts are made to separate these organisms from the host cells in which they have been grown, it is often found that more than half of the total infectious yield remains associated with intact host cells or host cell debris from which it can be separated only by mechanical disintegration or enzymatic digestion (see, for example, reference 475). It is not known if release is comparably imperfect in natural infections or if incomplete release is ever a limiting factor in the natural transmission of disease.

Modification of intracellular parasites at time of release. Many viruses are profoundly modified when their nucleocapsids are enclosed by membrane-containing envelopes acquired as they bud from virus-modified patches of plasma membrane (379). Although there seems to be no comparable widely occurring mechanism among organismic intracellular parasites, some of them are also modified as they leave their host cells. The enclosure of *R. tsutsugamushi* in host membranes during release (117) most closely resembles viral envelopment. It would be interesting to know if the exiting rickettsiae also acquire specific, parasite-modified portions of the plasma membrane of their host cells. *Coxiella burnetii* cells released from baby hamster kidney cells by spontaneous lysis or mechanical disruption show differences in incorporation of precursors into macromolecules (475) which may represent release-associated modification, although other explanations are possible. A final example of alteration at release has already been described in other contexts (see sections, "Entry" and "Multiplication"). In *C. psittaci*, the extensive disulfide bond cross-linking of the major outer membrane protein characteristic of extracellular elementary bodies occurs only when the elementary bodies pass from the relatively reducing intracellular milieu into the relatively oxidizing exterior. Cross-linking may be essential for the infectivity and durability of the chlamydia elementary body.

TRANSIT

Two kinds of transit may be visualized: passage of an intracellular parasite from one host cell to another, either in a cell culture or in a multicellular host, and passage from one intact host to another. In both kinds of transit, intracellular parasites are *r*-strategists in that they rely on production of large numbers of offspring. However, most of them have also evolved special devices, such as environmentally resistant transit forms or dependence on arthropod vectors, for increasing the chances of successful passage from cell to cell and from host to host.

Cell-to-Cell Transit

Transfer without intervention of an extracelullar phase. When *C. trachomatis* and *C. psittaci* infect L and HeLa cells in multiplicities of one to five chlamydiae per host cell, the infected cells continue to grow and divide, and often both daughter cells contain chlamydial inclusions (44, 197). Because the multiplicity of infection of new cells in intact hosts is probably often very low, this mode of cell-to-cell transit may offer a significant avenue of spread for many intracel-

TABLE 3. Transit-facilitating adaptations (no arthropod vector)

Parasite	Multiplication cycle	Infectious forms		Reference(s)
		Cell to cell	Host to host	
Bdellovibrio bacteriovorus	Motile rod ↑ ↓ Nonmotile filament	Motile rod		350, 393
Chlamydiae	Elementary body → Reticulate body ↑ ↓ Reticulate body → Elementary body	Elementary body	Elementary body	290
Coxiella burnetii	Sporelike body → Vegetative body ↑ ↓ Vegetative body → Sporelike body	Vegetative body	Sporelike body	29, 263, 451
Microsporidia	Meront → Spore ↑ ↓ Schizont	Meront	Spore	308, 427
Toxoplasma gondii	*In cats*: Oocyst → Sporozoite → Trophozoite → Schizont → Gametocytes → Gametes → Oocyst; Sporozoite → Schizont → Merozoite; Trophozoite → Schizont → Pseudocyst. *In other mammals and birds*: Trophozoite → Schizont → Merozoite; Trophozoite → Schizont → Pseudocyst	Trophozoite, merozoite	Oocyst, pseudocyst	131, 208, 308

lular parasites, particularly in tissues with rapidly dividing cell populations.

Transfer via an extracellular phase. Consideration of this mode of transit in cell cultures evokes a feeling of déjà vu. The infective cycle has been completed, we are just looking at entry from a different angle, and little new can be said. Intracellular parasites released into the cell culture medium appear to find new cells by random contact. Anything that increases frequency of contact, such as agitation of parasites and host cells or increasing host cell density, usually increases the chance of successful cell-to-cell transit. However, the efficiency with which different intracellular parasites turn random contact into successful entry varies tremendously, as I have already indicated above.

In intact hosts, intracellular parasites appear to spread from cell to cell by the same general routes followed by other pathogens (276). In brief, they move from cell to cell in the fluids covering epithelial layers and surrounding more deeply situated cells. They may reach the bloodstream directly or, more often, by way of the lymphatic system.

Some intracellular parasites, such as *Toxoplasma gondii*, a relative of the malarial parasites and a frequent parasite of humans, other mammals, and birds (120), *T. cruzi* (55), and avian strains of *C. psittaci* (270), infect a wide variety of host cells, whereas others show a strong predeliction for growing in one organ or tissue, sometimes in a single cell type. Erythrocytic merozoites of malaria infect only erythrocytes (141), *Rickettsia* species grow almost exclusively in the endothelial cells lining the small blood vessels (449), the trachoma biovar of *C. trachomatis* prefers the squamocolumnar epithelial cells of the genital tract and the conjunctiva (290), and *S. flexneri* multiplies chiefly in the epithelial cells of the colon wall (127, 128), to give only a few examples. It is not known whether these cells are reached with exceptional efficiency or whether they provide exceptionally favorable intracellular habitats. Growth of *M. tuberculosis* (472) and *Leishmania* species (78) occurs mainly in macrophages, although leishmanias also enter nonprofessional phagocytes (245). *Brucella abortus*, a facultative intracellular bacterium that causes abortion in cattle, grows preferentially in the bovine placenta because that tissue contains an unusually high concentration of erythritol, a growth factor for *Brucella abortus* (316). *Coxiella burnetii* also grows luxuriantly in ovine placenta, and several major laboratory outbreaks of Q fever have been traced to experiments with pregnant sheep with inapparent (but highly transmissable!) infections (29). However, why *Coxiella burnetii* has a predeliction for placenta is yet to be determined.

Host-to-Host Transit

Spread of infection from host to host can occur either horizontally or vertically (153). In horizontal spread, the intracellular parasite passes postnatally from one individual to another. Transfer may be direct and uncomplicated, as in the contraction of Q fever (29) or psittacosis (270) by inhalation of parasite-laden aerosols or in the acquisition of shigellosis by ingestion of fecally contaminated food or water (354). However, other intracellular parasites, such as *Plasmodium*, *Rickettsia*, and *Leishmania*, pass from host to host only with the help of arthropod vectors.

In vertical transmission, one of the parents, usually the mother, infects the offspring, either in utero or at the time of birth. When a pregnant woman has a genital infection with *C. trachomatis* (biovar trachoma), there is a strong possibility that she will pass the infection to her child at parturition so that it develops a chlamydial conjunctivitis or pneumonia (362). Toxoplasmosis is transmitted both vertically and horizontally. In vertical transmission, *Toxoplasma gondii* from a pregnant woman with parasitemia passes across the

TABLE 4. Transit-facilitating adaptations (with arthropod vectors)

Parasite	Vertebrate host			Arthropod host			Specialized infectious form	Reference
	Multiplication cycle	Infectious forms: Vertebrate cell to vertebrate cell	Infectious forms: Vetebrate to arthropod	Arthropod	Multiplication cycle	Infectious form: arthropod to vertebrate		
Leishmanias	Amastigote ↑ ↓ Amastigote	Amastigote	Amastigote	Sand flies	Amastigote → Promastigote ↑ ↓ Promastigote	Promastigote	+[a]	308
Trypanosoma cruzi	Amastigote → Trypomastigote ↑ ↓ Amastigote	Amastigote	Trypomastigote	Reduviid bugs	Trypomastigote ↑ ↓ Epimastigote ↑ ↓ Epimastigote	Trypomastigote	+	308
Plasmodia	Merozoite → Gametocytes; ↑ Trophozoite; Schizont	Merozoite	Gametocytes	Mosquitoes	Gametocytes → Gametes ↓ Oocyst ← Zygote ↓ Sporozoites	Sporozoite	+	141
Rickettsiae	No morphologically specialized cell types have been demonstrated			Ticks, fleas, mites	No morphologically specialized cell types have been demonstrated		0	449

[a] *Leishmania* promastigotes differentiate into an infectious stage that does not have a readily apparent morphological identity (358).

blood-fetal junction in the placenta and infects the late fetus to produce severe central nervous system damage or even stillbirth (120, 130), whereas horizontal transmission to adults, probably by ingestion of oocysts from cat feces (131, 208), usually results in only subclinical infection.

Adaptations that Facilitate Transit

In detail, adaptations that facilitate transit vary greatly from parasite to parasite, but in general they are made by splitting the conflicting demands of intracellular multiplication and extracellular transit beween two or more phenotypes so that survival as a species is assured by alternation of parasite cell types and often by alternation of host as well. In different host-parasite systems, these alternations are called by different names: growth cycle, developmental cycle, and life cycle, for example. They most commonly consist of differentiation into reproductive and infectious forms or utilization of arthropod vectors. Tables 3 and 4 summarize representative transit-facilitating adaptations made by both procaryotic and eucaryotic parasites. Each has been described in more detail at the first text reference to that parasite.

Differentiation into specialized reproductive and infectious forms. This division is exemplified by the filaments and motile rods of *Bdellovibrio*, the reticulate bodies and elementary bodies of *Chlamydia*, and the schizonts and merozoites of *Plasmodium*. Specialized infectious forms may attach to and enter host cells with unusual efficiency (such as merozoites, elementary bodies, and motile bdellovibrios), they may resist extracellular inactivation to an unusual degree (such as the pseudocysts and oocysts of *Toxoplasma*), or they may do both (such as microsporidian spores). Specialized infectious forms have evolved with (*Plasmodium*, *T. cruzi*) or without (*Chlamydia*, *Coxiella*, *Toxoplasma*) accompanying adaptation to arthropod transmission. When an intracellular parasite uses an arthropod vector, a cell type specially adapted to infect the vector is sometimes (malarial gametocytes, *T. cruzi* trypomastigotes) but not always (rickettsiae) produced. Specialized reproductive forms are adapted to doing just that in the intracellular habitat. The ability of reticulate bodies (but not elementary bodies) of *C. psittaci* to exchange a molecule of chlamydial ADP for a molecule of host ATP (37, 178, 179) furnishes a rare example of what reproduction-favoring adaptation may be like.

Utilization of arthropod vectors. Special infectious forms facilitate both cell-to-cell and host-to-host transfer, but arthropods only transport intracellular parasites from one host to another. There are several advantages to having an arthropod vector. The parasite is not exposed to a hostile nonliving environment, it usually mutiplies manyfold in its vector, thus augmenting the original population of parasites that infected the arthropod, and the feeding habits of the vector give the augmented population a more than random chance of reaching a new host. There are many variations in the details of arthropod transmission which can be illustrated by taking two extreme examples. The spotted fever agent *R. rickettsii* is a parasite of ticks in which it is passed from generation to generation by the transovarian route (64). People become accidental, blind alley hosts when they happen to be bitten by an infected tick. On the other hand, under natural conditions, human malaria requires the obligate participation of both humans and mosquitos (141). Plasmodia multiply asexually in people (merozoite → schizont) where they also produce sexual forms (gametocytes) that mature (gametes) and fuse (zygote → oocyst) only in mosquitos in which large number of human-infectious forms (sporozoites) are produced.

Other adaptations. Although chlamydiae are not known to use arthropod vectors, *C. trachomatis* uses people as both hosts and vectors; that is, it is sexually transmitted, a highly efficient adaptation in which the sexual drives of the host are exploited for transmission of the parasite (362). The wide distribution of the legionnaires' bacterium *L. pneumophila* in aquatic habitats despite its exacting growth requirements in

the laboratory (238, 404) has led investigators to search for an explanation of this paradox. It has been suggested that legionellae may use extracellular products of cyanobacteria or other aquatic microorganisms as growth factors (405) or that they may multiply and persist inside freshwater amoebae (189, 353) or ciliates (124). If proven, either mechanism of extracellular survival would be, in a broad sense, another example of facilitation of transit.

EVOLUTION OF INTRACELLULAR PARASITISM

Convergence and Divergence Among Intracellular Parasites

Convergence. It has been possible to describe adaptation to intracellular habitats by a variety of unrelated procaryotic and eucaryotic parasites within a single framework because of convergence, the evolution of similar adaptations among distantly related organisms living in similar environments (91). Time after time, the same end is achieved by different means. For example, *M. tuberculosis*, *C. psittaci*, and *L. pneumophila* all have learned to avoid intracellular destruction by not provoking phagosome-lysosome fusion (see section, "Survival"), yet for each parasite the precise mechanism of avoidance seems to be different. For two intracellular parasites sitting on different limbs of the phylogenetic tree to share an adaptation of common origin, it would have to be conserved as it was passed along by many intermediate organisms in extracellular habitats, a most unlikely series of events.

Divergence. In large groups of intracellular parasites of known or presumed close evolutionary relationship, evolutionary diversification, or adaptive radiation (91), has occurred when parasites of common origin have adapted to different hosts and different ways of getting from host to host. Several examples of adaptive radiation have already been described. However, since they were not designated as such, a brief recapitulation may be useful.

The genus *Rickettsia* comprises a large group of organisms closely related genotypically (295) and with strong phenotypic resemblances (449). They resemble each other closely in patterns of metabolism, but they differ in modes of egress from host cells (see section, "Release") and in choice of arthropod vector (see section, "Transit").

The genus *Plasmodium* represents an even larger assemblage of intracellular parasites which are conventionally related one to another largely in terms of their vertebrate hosts (141), a classification that does not always fit recent estimates of genetic relatedness (264). There are metabolic differences among the erythrocytic stages of different species, but they are largely second-order: the grand pattern is the same. Plasmodia all appear to enter erythrocytes by the same general mechanism, and they have stuck with the mosquito as their arthropod vector. Main differences appear in the life cycle, whether there are (*P. vivax*) or are not (*P. falciparum*) resting forms (hypnozooites) in hepatocytes (reviewed in reference 428), and in behavior of parasitized erythrocytes in the vertebrate vascular system, whether there is (*P. falciparum*) or is not (*P. vivax*) retention of large schizonts in the capillaries of the internal viscera (141).

The two chlamydial species, *C. trachomatis* and *C. psittaci*, show strong phenotypic resemblances down to the molecular level but only marginal genetic similarity (288). There are different entry mechanisms, but the separation is not along species lines: the LGV biovar of *C. trachomatis* resembles *C. psittaci* more than the trachoma biovar of the same species. Differences in chlamydial amino acid requirements are nicely explained by adaptive radiation. *C. psittaci* needs only 8 (31) of the 12 essential amino acids of Eagle medium (105), whereas the LGV and trachoma biovars of *C. trachomatis* require all 12 essential amino acids plus serine (311). Within a single species (*C. psittaci*), isolates of different host origin do not have exactly the same amino acid requirement, and within a single biovar (trachoma), strains isolated from different human clinical conditions need different sets of amino acids (17).

Several other groups of intracellular parasites, some facultative and others obligate, would almost certainly provide more good examples of adaptive radiation if only more were known about them. Leishmanias, legionellae, and the gram-negative enterics come readily to mind.

Transition from Extracellular to Intracellular Life

A parasite takes a big jump in trading the extracellular way of life for an intracellular one, a jump comparable to Darwin's (92) example of an animal learning to fly. On first thought, they both appear to be discontinuous changes: fly or not fly, grow inside cells or not grow. If, as I have argued in this review, success in the intracellular habitat depends on a number of independent adaptations, the likelihood of any organism making the jump does not seem great. For *S. flexneri* (127, 128, 163) and *Y. pestis* (61), in which the minimum number of these adaptations can be estimated precisely by genetic analysis, the number is four and six, respectively. Loss of any one of these results in loss of virulence which seems to be the same as loss of ability to live intracellularly. However, since intracellular parasites have evolved many times in many different phylogenetic lineages, we must conclude that it is not so hard to become an intracellular parasite after all. Perhaps the transition from extracellular to intracellular existence is indeed a large evolutionary jump, but the inside of a living cell is an environment that favors the occurrence of this unlikely event. On the other hand, it may be that the shift to intracellular life need not be as discontinuous as it first appears. The two possibilities are not mutually exclusive.

Possibility of large evolutionary jumps. E. B. Ford has said, "There are a number of situations which tend to promote rapid evolution. One of these results from adaptation to a new and distinct habitat, in which, if a species can survive, powerful selection will be operating to promote appropriate modification to its new environment" (126). The "new and distinct habitat" is the inside of a living cell, and "powerful selection" is operating because only the would-be intracellular parasites that escape their host cells to reach and survive in new cells reproduce their own kind. It has been suggested that species-poor, stressed environments favor the survival of evolutionary novelties and perhaps even enhance the likelihood of big evolutionary jumps (210). In another context (282, 283), I have pointed out the resemblance of intracellular habitats to conventional extreme environments which are both stressed and species poor. There is at least one laboratory demonstration of how fast adaptation to intracellular life can occur (213). A gram-negative rod, probably a member of the bacterial family *Enterobacteriaceae*, progressed from being a lethal parasite for *Amoeba proteus* to being a required host cell component of that protozoan in only 200 generations.

Among the procaryotic pathogens, virulence genes may be carried on either chromosomes or plasmids, which are extrachromosomal hereditary elements consisting of autonomous, self-regulating DNA molecules that confer unique properties on the host in which they reside (112). Acquisition

of a plasmid carrying a gene whose expression improves intracellular adaptation might well constitute a big evolutionary jump. At least two of the five virulence determinants of *Y. pestis* (61, 122) and one of *S. flexneri* (359) are encoded by plasmid genes. Other intracellular parasites such as *Coxiella burnetii* (257) and chlamydiae (250) also contain plasmids, but their contribution to successful intracellular life is not known.

Possibility of gradual adaptation. A consideration of the relation of symbiotic algae to their hosts led to the conclusion that there is no sharp transition from extracellular to intracellular but rather a graded series of relationships (380). For example, algal cells are truly extracellular to fungal cells in lichens, surrounded but not inside of host cells in some alga-flatworm symbioses, and completely inside host cells in the *Hydra-Chlorella* symbiosis. In host-parasite relationships, there are abundant examples of truly extracellular and truly intracellular configurations, but examples of intermediate topology are rare. *Cryptosporidium* is the best. It is an intestinal sporozoan distantly related to *Plasmodium*, less distantly to *Toxoplasma* (298, 420). Unlike other sporozoans, which all grow intracellularly, it multiplies attached to its host cell but not truly inside. The cytoplasm of the cryptosporidian trophozoite is enclosed in four membranes, and the outer two appear to be of host origin (43, 326, 429). Cryptosporidia, therefore, seem to multiply intracellularly but not intracytoplasmically, a situation roughly analogous to the intraperiplasmic location of bdellovibrios in their hosts.

The abruptness of the extracellular-intracellular transition may also be smoothed out by preadaptation, the evolutionary acquisition of a character with survival value in one situation which is later of value for a different reason in a different set of circumstances (91). What immediately comes to mind is the frequently encountered adherence of extracellular bacterial pathogens to specific kinds of host cells (38). Both cholera vibrios and shigellae adhere to the intestinal mucosa, but only shigellae invade (128). If some rogue vibrio decides to invade the intestinal wall, it is already preadapted for the first step in entry, attachment.

The unexpectedly good survival of the legionnaires' bacterium in freshwater habitats has just been offered (see preceding section) as a possible example of an unusual transit-facilitating strategy of an established intracellular parasite, but suppose that *L. pneumophila* is not an established parasite, that its true habitats are ponds, cooling towers, and showerheads. Then, when it infects a human, this is a first-time contact for that legionella and all of its progenitors. Is it preadapted for intracellular life? Entry and survival adaptations have already been demonstrated (see section, "Survival"). *L. pneumophila* enters nonprofessional phagocytes, and it does not provoke lysosomal fusion in professional ones. Which view of the legionnaires' bacterium is correct? The observation that only a small fraction of the legionellae ingested by primate alveolar macrophages survive and multiply intracellularly (212) can be interpreted either way. It is the chicken-or-the-egg question all over again.

Passage from extracellular to intracellular habitats may be eased in yet another way. It may be that an extracellular parasite needs to make only minimal adaptation to gain an intracellular entree and that additional adaptations are made thereafter. The familiar intracellular parasites described here are all highly successful, fully adapted residents of host cells. Their less well-adapted ancestors will have been replaced long ago by these more efficient descendents. This is a frequent evolutionary argument (see, for example, reference 339) which does not warrant elaboration, but it is worthwhile to ask if any known pathogen might serve as a paradigm. *Neisseria gonorrhoeae* might just fit the bill (265, 439). This sexually transmitted gram-negative coccus first attaches to the microvilli of columnar epithelial cells in patients with gonorrhea and in human fallopian tube organ cultures and then to the larger surface areas of epithelial cells. Once firmly attached, the gonococcus multiplies extensively on the host cell surface, and a very small fraction of the adherent organisms is taken into epithelial cells by what appears to be phagocytosis (although these cells are not professional phagocytes). There they are to be found in membrane-bound vacuoles that do not precipitate lysosomal fusion. Finally, the membrane-enclosed gonococci migrate to the basal portions of their host cells where they are released by cell lysis or extrusion of entire vacuoles, and infection spreads laterally along the subepithelial connective tissue. Whatever its present function in gonococcal infection, this migration of the gonococci through the epithelial cells of the urogenital tract could conceivably provide a jumping off place for evolution of a strictly intracellular *Neisseria* sp.

An incompletely adapted parasite could also get started on intracellular life by being phagocytized by a macrophage and surviving (preadaptation?) long enough to make further adaptations to intracellular life, even to the point of learning how to enter nonprofessional phagocytes. The observation that the exoerythrocytic forms of avian malaria, thought to be the oldest of the malarias, inhibit liver macrophages while those of mammalian malaria prefer hepatic cells (141) might be interpreted as confirmation of this conjecture.

Relatives of intracellular parasites among modern extracellular parasites. If intracellular parasites have actually descended from extracellular ones, then extracellular relatives of present day intracellar parasites should be recognizable (164). A case can be made for *Rochalimaea quintana* (450), the trench fever agent, being such a relative. *R. quintana* and the typhus rickettsiae engage in DNA-DNA hybridization to the extent of 25 to 33% (295). Like rickettsiae, the trench fever agent catabolizes glutamate but not glucose (447). Unlike rickettsiae, it grows on nonliving media (431), but in association with eucaryotic cells it grows profusely on their surfaces (269). *T. cruzi* offers an opposite kind of example. The genus *Trypanosoma* contains several species infecting humans, all extracellular, blood-dwelling parasites except *T. cruzi* (83). The trypomastigote of *T. cruzi* is morphologically similar to the blood forms of *T. brucei* and *T. gambiense*, the agents of African sleeping sickness, but unlike these trypanosomes the *T. cruzi* trypomastigote does not multiply in the human bloodstream (Table 4).

DIFFERENCE BETWEEN OBLIGATE AND FACULTATIVE INTRACELLULAR PARASITES

Obligate intracellular parasites are restricted to an intracellular way of life, whereas facultative intracellular parasites are not subject to this restriction: they also multiply in environments totally devoid of host cells. Obligate intracellular parasitism appears to be just one example of a broader phenomenon. Microorganisms that have adapted to growth in extreme environments such as salt lakes and hot springs are also often restricted to those habitats (14). The restriction of some intracellular parasites to the intracellular habitat, which has many resemblances to conventional extreme environments (282, 283), may have a comparable explanation. In learning to grow inside host cells, obligate intracel-

lular parasites have either gained new characters that are inimical to multiplication extracellularly or lost old characters that are essential. Perhaps they have done both. In contrast, facultative intracellular parasites have adapted to intracellular life without losing the ability to grow extracellularly.

A number of writers, including myself, have spent much time in speculating on why obligate intracellular parasites do not multiply outside of host cells. However, I have come to believe that undue preoccupation with this question is unwarranted because the answers are likely to be trivial and unrelated to the far more important question, why intracellular parasites, obligate and facultative alike, do grow inside of host cells.

Growth of Facultative Intracellular Parasites in Host Cell-Free Media

The relatively simple growth requirements of many facultative parasites are consistent with the interpretation of facultative and obligate ways of intracellular life I have just given. For example, *S. flexneri* grows well in a synthetic medium containing only aspartate, glucose, salts, nicotinic acid, and thiamine (115). Another facultative intracellular parasite, *M. tuberculosis*, the human tubercle bacillus, also grows in simple synthetic media. Glycerol and ammonium salts are sometimes all that are absolutely required, but asparagine, other amino acids, and biotin sometimes promote more rapid growth (227). Even *L. pneumophila*, which is often thought of as having exacting growth requirements, has been serially transferred in a defined medium containing only inorganic salts and nine amino acids (399). It is clear that these organisms are facultative, not obligate, intracellular parasites because they have managed to adapt to life inside cells without material reduction in their biosynthetic capabilities.

It is possible that some obligate intracellular parasites have so completely adapted to the intracellular habitat that they will be persuaded to multiply in cell-free environments only when near-complete intracellular habitats are reconstructed in vitro. However, it is more likely that there is no discontinuity between the growth requirements of what are now classified as facultative or obligate, that the dividing line will shift with the state of the cultivation art, and that eventually there will be no more obligate intracellular parasites. The dividing line has shifted, but not easily. Thirty-five years ago, it was demonstrated that blood stages of the malarial parasite *P. lophurae* remained in good condition outside the erythrocyte for as long as 3 days and developed from small, mononucleated forms to large, multinucleated stages in a growth medium containing erythrocyte extract, pyruvate, B vitamins, and ATP (408). However, despite long-continued efforts, significant extracellular multiplication of *P. lophurae* has still not been achieved (414). Although most mycobacteria grow in cell-free media, the leprosy bacillus, *M. leprae*, has obstinately resisted all efforts to grow it in vitro, efforts that have continued unabated since soon after its discovery in 1873 (165). In contrast, the agent of rat leprosy, *M. lepraemurium* (20% DNA relatedness to *M. leprae* [26]), which remained an obligate intracellular parasite for many decades, has now been grown in vitro in media containing salts, citrate, glutamate, glycerol, pyruvate, α-ketoglutarate, hemin, cysteine, and cytochrome *c* (97, 98, 296, 297).

Unique Uses of Facultative Intracellular Parasites

Nothing I have just said should be taken as minimizing the importance of propagating every intracellular parasite in host cell-free media at the earliest opportunity. Host-independent cultivation provides large numbers of parasites free of host contamination for biochemical and immunological investigations. If the parasites grown outside of host cells prove different from those grown inside, the differences may well provide valuable insights into the influence of the intracellular habitat on parasite genotype and phenotype. The ability to grow an intracellular parasite outside of the host cell also provides an opportunity to analyze the presumed multifactorial nature of adaptation to intracellular life. If a parasite can be propagated only inside host cells, then loss of only one character vital to intracellular life is lethal, that character cannot be identified, and no analysis is possible. However, if the defective parasite can still grow extracellularly, the lost character can be identified, and the analysis can continue.

CELL CULTURES INFECTED WITH INTRACELLULAR PARASITES AS MODELS OF INFECTIOUS DISEASE

Since a large fraction of the results discussed in this review were obtained with populations of infected host cells maintained in vitro, it is appropriate to ask what pertinence these results have to naturally acquired infections in natural hosts. In theory, cell cultures infected with intracellular parasites are valid models of infectious disease because, as a first approximation, the effect of an intracellular parasite on its multicellular host is the sum of its effects on all infected cells in that host. In practice, the usefulness of any particular model depends on just how good the approximation is.

Limitations of Cell Culture Models

Models are of limited usefulness when they fail to duplicate some essential element of the phenomenon being modeled. Several examples come to mind.

Dosage. Although, under natural conditions, most hosts are infected with very low doses of intracellular parasites, cell cultures are usually infected with unnaturally large inocula to obtain uniformly infected host cell populations for which doubtful corrections for the activity of uninfected cells need not be made. However, as with chlamydiae and L and HeLa cells, multiplicity of infection is often the dominant determinant of the outcome of the host-parasite interaction in vitro. At very low multiplicities, the host cell is not critically injured and may pass through several cell divisions (90, 44, 197), but at high multiplicities it is killed within a few hours (289).

Architectural complexity. Cell cultures are either suspensions or monolayers of a single cell type, whereas intact hosts are collections of many cell types gathered together in different proportions in three-dimensional tissues and organs. If the fate of an infected host cell is influenced by the kind and number of adjacent cells, then the cell culture model may be inadequate. However, one architectural parameter that may be studied in cell culture is population density. The concentration of isoleucine needed to initiate multiplication of *C. psittaci* in L cells increases with increasing L-cell density (176), and the regulation of overt chlamydial multiplication in persistent infections of McCoy cells with *C. trachomatis* (240) and L cells with *C. psittaci* (287) is in part a function of host cell density. There are probably more complex density-dependent regulatory relationships in intact hosts. Perhaps they can be studied in organ culture.

Hormones, growth factors, antibodies, and lymphokines. In the intact host, the behavior of an infected cell is modified not only by the other host cells in its immediate vicinity, but

also by distant cells which secrete chemical signals that are carried to it in the blood. These signals are the hormones and growth factors secreted by a variety of cell types, the antibodies secreted by B lymphocytes, and the lymphokines liberated by T lymphocytes. An infected cell will not model the effect of a signal if the signal is not there. If the identity of the signal is known, it may be added to the cell culture. A striking example of how the usefulness of cell culture models may be extended in this way is the recent demonstration (discussed below) that interferon-γ added to cultures of both professional and nonprofessional phagocytes activates the host cells so that the intracellular multiplication of a number of parasites is inhibited.

Contribution of Cell Culture Models to the Comparative Biology of Diseases Caused by Intracellular Parasites

If cell culture models are to contribute to the comparative biology of infectious disease, they must reproduce the phenomenon being compared in enough different intracellular parasite-host cell systems to make the comparison worthwhile. Within limits, both parasite virulence and host resistance may be modeled in cell culture. The following examples illustrate the value and limitations of cell culture models.

Host range. If there is a correlation between host range in vivo and in vitro, then the determinants of host range in naturally acquired infections may be analyzed in cell culture. The E strain of *R. prowazekii* has been attenuated by passage in chicken embryo yolk sac so that it has a lessened capacity to infect humans and other animals (see reference 129) and to grow in human macrophages (139). Reports of its behavior in cell culture are contradictory. The attenuated strain grows less luxuriantly than a virulent strain in a monkey kidney cell line (21), but it enters and multiplies in chicken embryo cells at the same rate as the same virulent strain used in the other study (466). In L cells, the E strain grows as well as virulent *R. prowazekii*, but in several macrophage-like cell lines it does not grow as well as the virulent strain (418).

The relation between host range of chlamydiae and behavior in cell culture was discussed in the section, "Entry". In brief recapitulation, natural infections with *C. psittaci* occur in many orders of birds and mammals, whereas people are the only natural hosts for the trachoma and LGV biovars of *C. trachomatis*. *C. psittaci* multiplies to high titer in many kinds of cell cultures, but the trachoma biovar multiplies in relatively few cell types and with difficulty even in them. However, the LGV biovar spoils the correlation between host range and in vitro behavior. Despite its limited natural range, it grows just about as well as *C. psittaci* in cell culture.

In malaria, the vertebrate range of *P. knowlesi* (275) and *P. falciparum* (69, 182) correlates well with the ability of erythrocytic merozoites to enter and multiply in erythrocytes of different species maintained in vitro, this despite the fact that the first vertebrate cells to be invaded are the hepatocytes (by sporozoites), not erythrocytes. Recent successes in infecting cell lines and primary hepatocyte cultures with sporozoites of rodent and human malarias (see reference 260) will permit further test of the correlation. Taken together, all of these findings suggest that some, but by no means all, of the determinants of natural host range may be modeled in infected host cells cultured in vitro.

Host injury. Damage to host cell populations maintained in vitro by infection with *Coxiella*, *Rickettsia*, *Chlamydia*, and *Plasmodium* species has already been noted (see sections, "Entry" and "Multiplication" and the summary in Table 2). In vitro host cell injury will now be reexamined by asking if it helps to understand the pathogenesis of diseases caused by these organisms in intact hosts. Not much is known about the pathogenesis of Q fever (*Coxiella burnetii*) in humans, but the pathology of the disease in guinea pigs has been studied exhaustively (29). The main target is the liver, in which steatogenesis, glycogenolysis, increased protein and RNA syntheses, and increased phosphorylation of nuclear proteins occur. Although *Coxiella burnetii* multiplies extensively in L cells, host cell injury is minimal, and persistent infections are easily established. Glucosamine incorporation is decreased in plasma membranes from infected guinea pigs and increased in membranes from infected L cells (258). Perhaps the use of hepatocyte cultures instead of L cells would improve the correlation between in vivo and in vitro effects of infection.

The pathogenesis of rickettsial disease may be ascribed in large part to infection of the endothelial cells of the vascular system (433). Although the effect of rickettsiae on host cells in vitro has been studied in the past with fibroblastic lines such as L cells, it has recently been demonstrated that both *R. rickettsii* (374, 375) and *R. prowazekii* (435) enter cultured human endothelial cells in ways closely resembling the entry of these organisms into L cells. Therefore, it is likely that endothelial cells are injured in vivo, at least in part, by the mechanism already demonstrated in the L-cell model: plasma membrane damage by phospholipase A at the time of rickettsial attachment (461, 462).

Unlike rickettsiae, chlamydiae infect a variety of cells in their natural hosts (290). *C. psittaci* infects many kinds of cells, *C. trachomatis* (biovar LGV) prefers lymphoid cells, and *C. trachomatis* (biovar trachoma) grows mainly in squamocolumnar epithelial cells, and the pathogenesis of chlamydial disease cannot be broadly explained in terms of a single host cell target. Like rickettsiae, chlamydiae damage host cells during entry (see section, "Entry"). In vitro, the multiplicity of infection required for immediate host cell injury ranges all the way from 10 in mouse peritoneal macrophages (M. Gardner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, D6, p. 70) to 500 for L cells (289). The sensitivity of naturally infected host cells is unknown.

In malaria, there is no doubt as to the nature of the target cell, and both the breakdown of hemoglobin and the alteration of the erythrocyte surface described in the section on "Multiplication" occur in parasitized erythrocytes maintained in vitro (413). Even the binding of erythrocytes infected with late stages of *P. falciparum* to the capillary endothelium by means of the knoblike protrusions on the erythrocyte surface is reproduced in cultures of human endothelial cells (421).

The general conclusion is that with some intracellular parasites, such as rickettsiae and malarial parasites, there is good correlation between host damage in vitro and in vivo and that with others, such as Q fever organisms and chlamydiae, there is not. In infections caused by the latter two parasites, important determinants of pathogenesis may be lacking in the cell culture model.

Immune response. Many aspects of the interaction of intracellular parasites with intact immune hosts are too complex to be reproduced in cell culture (see reference 106). However, two immune phenomena that can be studied in vitro and are also of interest from the comparative viewpoint are the effects of antibody and interferon-γ on entry and multiplication.

With the aid of monoclonal antibodies and in vitro culture methods, antibodies have been shown to block virtually every stage in the malarial life cycle (see Table 4 for an outline of the life cycle of malarial parasites). When sporozoites of *P. berghei*, *P. vivax*, and *P. falciparum* invade hepatoma cells in vitro, their entry is blocked by species-specific monoclonal antibodies against the circumsporozoite protein (190, 191). The entry of merozoites of *P. knowlesi* into cultured rhesus erythrocytes is inhibited by monoclonal antibodies to surface antigens of 66,000 (95) and 140,000 (141, 274) molecular weight. It has been suggested that these antibodies do not cover merozoite receptors for erythrocytes, but that instead they agglutinate the merozoites before they encounter uninfected erythrocytes (274). Monoclonal antibodies to zygotes of *P. gallinaceum* block the infectivity of gametes for mosquitos either before (216) or after (154) fertilization. The target of the antibodies that block before fertilization has not been identified, but the target of the antibodies that block transmission after fertilization is a surface antigen of the zygote with a molecular weight of 26,000 (154). It seems likely that antibodies that disrupt the several stages of the malarial life cycle act chiefly by binding to surface antigens of the plasmodial cell and hindering its entry into an appropriate host cell, although the possibility of inhibition of intracellular multiplication cannot be ruled out.

Antibody also neutralizes chlamydial infectivity for cell cultures. Antibody against intact *C. trachomatis* (LGV) and *C. psittaci* blocks infection of nonprofessional phagocytes at the attachment stage when elementary bodies attach spontaneously (6, 18, 73, 438), but when the chlamydiae are centrifuged onto host cell monolayers (see section, "Entry"), neutralization occurs at some stage beyond attachment (6), and the same antiserum that blocks attachment of *C. psittaci* to L cells (nonprofessional phagocytes) accelerates its association with mouse macrophages (professional phagocytes) (M. Gardner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, D6, p. 70). When the purified chlamydial major outer membrane protein (see section, "Multiplication") is the immunizing antigen, neutralization of *C. trachomatis* (LGV) occurs after attachment and is independent of complement (74, 318). In contrast, neutralization of *C. trachomatis* (biovar trachoma) by antibody against whole chlamydial cells in baby hamster kidney cells is increased 100- to 1,000-fold by the addition of fresh guinea pig serum (205). This suggests that the chlamydiae are lysed in a complement-dependent reaction, but other interpretations are not excluded. It must be concluded either that more than one chlamydial antigen induces neutralizing antibody or that the combination of the same antibody and antigen under different conditions affects chlamydiae in different ways.

The effect of antibody on the entry and intracellular fate of rickettsiae in cultured host cells also depends on the kind of host cell and kind of rickettsia involved. For example, antibody blocks the entry of *R. tsutsugamushi* into chicken embryo cells (166) but increases it rate of entry into guinea pig macrophages (342). Similarly, antibody is without effect on the entry and growth of *R. prowazekii* in chicken embryo cells (468), but it facilitates its entry into human macrophages without enhancing subsequent intracellular destruction (39). Finally, antibody increases the rate of entry of *R. typhi* into human macrophages (140) just as with *R. prowazekii*, but the combination of *R. typhi* with antibody also increases its rate of intracellular destruction. The targets of neutralizing antibody for rickettsiae are not known.

Interferon-γ, produced chiefly by T lymphocytes in response to mitogens or antigens to which they have been sensitized, in addition to inhibiting viral multiplication (218) also inhibits multiplication of many organismal intracellular parasites. A by no means complete list includes *P. falciparum* (306), *Toxoplasma gondii* (323), *Coxiella burnetii* (417), *R. prowazekii* (419, 467), and *C. psittaci* (72, 352). As in inhibition of viral multiplication, interferon-γ has no effect on host-free parasites, and, for maximum effect, must generally be added to host cells before infection. However, as was suggested some time ago (430), it is unlikely that interferon blocks viral and organismal intracellular parasites by the same mechanism. It has recently been reported that interferon-γ inhibits the growth of *T. gondii* by inducing the host cell to degrade tryptophan, which is assumed to be required by toxoplasmas (322). Continued study of how interferon-γ inhibits the growth of such a diverse group of organisms will be most interesting. Are there different modes of action, one for each parasite, or is there one common interferon-γ-sensitive step that bridges the procaryotic-eucaryotic discontinuity?

CONCLUSION

Any proper sort of review should have a conclusion, a brief recapitulation of what the writer thinks is important and what he hopes his readers will agree with. The most important conclusion I can come up with is that there are no sweeping generalizations. Many microorganisms of diverse phylogenetic beginnings have adapted to intracellular life, each in its own unique way, and the sometimes remarkable resemblances in behavior among intracellular parasites are best ascribed to convergence in similar intracellular habitats rather than to divergence from a common origin. Finally, it should be remembered that adaptation to intracellular life, although by no means rare, is not easy. After all, most parasites still live extracellularly.

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